LIPID NANOPARTICLE-MEDIATED DELIVERY OF ENHANCED COSTIMULATION BLOCKADE TO PREVENT TYPE 1 DIABETES

By

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ABSTRACT

Type 1 diabetes (T1D) remains an untreatable autoimmune disease caused by the destruction of pancreatic beta cells by autoreactive T cells. Because of its complex etiology, many immunotherapy strategies have been investigated, but with disappointing results. Costimulation blockade, blocking the CD28 pathway via administration of CTLA4-Ig is a promising approach, but recent observations suggest its efficacy is antagonized by inflammatory factors. As antigen presenting cells malfunctions and aberrant accumulation of type 1 interferons are associated with T1D, we pose that inhibiting the signaling of inflammatory cytokines via Tofacitinib (Tofa), a JAK inhibitor, would enhance the efficacy of CTLA4-Ig to prevent T1D development. The objective of this study was to design the controlled and localized delivery of Tofa via implementation of biocompatible lipid nanoparticles, Nanostructured Lipid Carrier (NLC), and assess the immunomodulatory impact of this strategy.

We identified a specific composition of NLC that had negligible toxicity, could be readily taken up intracellularly by multiple immune cells, and had a favorable Tofa encapsulation efficiency. Live animal imaging using fluorescently-labeled NLC confirmed that these particles have the unique property of accumulating in lymphoid tissues. Moreover, when administrated via oral gavage, they bypassed first-pass metabolism and accumulated in spleen, pancreatic and mesenteric lymph nodes. *Ex-vivo*, Tofa is rapidly delivered by Tofa-NLC to mouse antigen presenting cells preventing their maturation. Short-term administration of Tofa-NLC via oral gavage at early or later stage in NOD mouse can promote a significant reduction of T1D onset and delay the development of the disease. The use of CTLA4-Ig at different dosage yet did not give any protective effect. Current works demonstrate a promising delivery vehicle in local

delivery of anti-inflammatory agent, which provide therapeutic effect on T1D, and the mechanism(s) behind is still underway.

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INTRODUCTION

- **Type 1 Diabetes and the Immunological Basis Behind It**

Type 1 diabetes (T1D) is an autoimmune disease caused by the destruction of pancreatic beta cells by autoreactive T cells. There are two phases of the disease. The first, insulitis, is demonstrated by the invasion of pancreatic islets by leukocytes. As disease progress, it becomes diabetes, at which the majority of beta cells (> 70%-80%) has been destroyed. At this phase, the islet is incapable of producing sufficient insulin to regulate blood glucose, leading to hyperglycemia, which serves as a diagnostic hallmark of the disease [1]. Another feature of T1D is the presence of beta-cell autoantigens reactive autoantibodies, such as those reactive to insulin, glutamic acid decarboxylase (GADA), zinc transporter 8 (ZnT8A), and insulinoma-associated autoantigen 2 (IA2A), and these autoantibodies can be present much earlier than symptomatic onset [2]. The exact cause of T1D is not clear yet, however, genetic factors have long been considered as the major contributor to the disease. Among numbers of genes known to affect disease susceptibility, the human leukocyte antigen (HLA) complex on chromosome 6 perhaps is the most important one, and two haplotypes in HLA class II region have been used as the principal susceptibility markers for type 1 diabetes. Other diverse genes are thought to contribute to the remaining genetic risk, yet as more studies are being conducted, it is appreciated that nongenetic factors, particularly environmental factors, also play a significant role in the development of the disease [3, 4].

The etiology of type 1 diabetes is very complicated as both innate and adaptive immune system are involved in this process. The most important role the immune system played is to distinguish and react accordingly towards self- and non-self antigen. In healthy individuals,

tolerance to self is maintained by the corporation of multiple regulatory mechanisms. Alterations in these pathways by either genetic or non-genetic drivers can lead to signals that break homeostasis of the immune system and diminish tolerance to self-antigens. Centrally, self-reactive T cells carrying autoantigen receptors are eliminated in the thymus at a very early stage via negative selection. However, some autoreactive T cells escape and enter the peripheral as this process is not perfect [5]. Peripherally, homeostasis is maintained by various regulatory mechanisms and damage can be prevented even with the existence of escaped self-reactive T cells. One of the mechanisms is the conversion of autoreactive T cells into anergic cells. To fully activate T cells, both antigen-specific signals delivered via T cell receptor (TCR) and costimulatory signals are required. Fully activated T cells can then divide and differentiate into effectors and regulators, participating into immune responses. Providing TCR engagement alone will lead T cell to a state called anergy, in which they are functionally inactive (i.e. reduced effector functions, proliferation, and cytokine production) but can remain alive for an extended period [6, 7]. Another mechanism, activation-induced cell death (AICD), utilizes Fas and Fas Ligands (FasL) interaction. Activation of Fas expressed on beta-cells by FasL expressed activated T cells can induce apoptosis and lead to deletion of peripheral activated T cells. It has been demonstrated that in both mice and human, defects in Fas or FasL can initiate the development of multiple autoimmune diseases [7-9]. The third mechanism prevents autoimmune reactions by suppressing self-reactive lymphocytes via regulatory T cells (Treg). There are various subsets of Tregs (i.e. type 1 Tregs and T helper 3 Tregs), and they are mainly characterized by the expression of forkhead box transcription factor P3 (Foxp3). It has been clearly shown that loss-of-functions mutations of Foxp3 can lead to early onset of T1D in human and deficiency of Treg cells in mouse model can accelerate T1D, both indicating that Treg plays

a critical role in maintaining self-tolerance and regulating the development of the disease. It is now known that the suppressive function of Treg is applied either directly on effector T cells by producing immunosuppressive cytokines such as IL-10 and TGF-β, or indirectly on DCs by inhibiting their maturation and function [7, 8, 10]. However, these regulatory mechanisms can be disrupted, leading to off balance of the immune system and potentially initiating responses against self-tissue.

Activation of innate immune cells is often a pre-requirement in this loss-of-tolerance process. Some populations of antigen presenting cells (i.e. dendritic cells (DCs)) as well as innate signaling pathways (i.e. type 1 interferon (IFN)) have been closely related to triggering autoimmune responses [5]. Conventional dendritic cells (cDCs) can facilitate the initiation of disease by presenting self-antigens released from the body, while plasmacytoid DCs (pDCs) do it by secreting large amount of interleukin 12 (IL-12) and type 1 IFNs. The correlation of type 1 IFN with the initiation of T1D has been demonstrated in studies, in which the incidence increases as the production of type 1 IFN by pDCs in pancreatic lymph node (pLNs) increases [8, 11]. Macrophages also play a pathogenic role in both onset and destruction phases of T1D. Early experiments showed blocking the influx of macrophages into pancreas can inhibit the development of diabetes, and recently this is thought to be related to the production of IL-12, IL-1β, tumor necrosis factor (TNF), and reactive oxygen species (ROS) [12, 13]. In addition, certain viral infections (i.e. enteroviruses) have been considered to promote on environment that activated the innate immune system and initiates T1D. Viruses can influence beta-cells by direct or indirect damage to the cells, thus enhancing the release of beta-cell antigens. Indirectly, the infection in pancreas caused by viruses can trigger the recruitment of innate immune cells and activate autoimmunity, which is recently thought to be related with type 1 IFN production [5, 8,

14]. Other potential environmental influences thought to be related to the disease including exposition to microbiome, climate change, and nutrition [15, 16].

With various immune cells and complex pathway involved and multiple regulatory mechanisms built, a healthy immune system is able to prevent attacking of self-tissues and provide self-tolerance. However, any breaking points within the system can potentially lead to the initiation of the disease. For type 1 diabetes to develop, multiple events are needed: diabetogenic T cells need to be activated, the response need to be strongly proinflammatory, and last but not least, the regulatory control against autoreactive responses need to be ineffective [10]. Antigen presenting cells (APCs) such as DCs are important for activation of anti-islet response. They first take up and process beta-cell derived antigens (released due to beta-cell death) within islet and then migrate to the draining pancreatic lymph nodes (PLNs), where they present antigens to beta-cell reactive T cells and activate them [1]. The full activation of T cells requires stimulation of TCR by major histocompatibility complex (MHC), which is facilitated by appropriate costimulatory signals as well as macrophages (through the secretion of IL-12) [8, 17]. Activated T cells can then migrate back into islets, destroying beta-cells by releasing pro-inflammatory agents such as IFN-γ, perforin and granzymes, resulting in the initialization of insulitis [1]. In addition of cytotoxic helper T cells, B cells, dendritic cells, and macrophages can also infiltrate into pancreatic islet and causing beta cell damage by producing, for instance, tumor necrosis factor (TNF), IL-1β, and nitric oxide [8]. Damages on beta-cells can be controlled by aforementioned regulatory mechanisms, yet if these mechanisms failed, the destructions will continue to expand and eventually progress to diabetes.

- **Current Treatments, Clinical Trials, and Research**

Worldwide, the incidence of type 1 diabetes has been increasing. Approximately 1.25 million American has type 1 diabetes, and about 40,000 people in the U.S. will be newly diagnosed each year. Much effort has been put into the clinical care of T1D to help improve the quality of a patient's life and clinical outcomes. Although numerous therapeutic options have been investigated and developed, type 1 diabetes, unfortunately, still remains a disease with no cure. Current existing treatments mainly focus on managing blood glucose level through administration of insulin. Patients depend on lifelong continuous insulin administration, and receive multiple daily injections, including rapid acting insulin with meals and continuous infusion of basal insulin [3]. Despite the utilization of various insulin delivery technologies (i.e. insulin pumps and continuous glucose monitors), many patients suffers devastating consequences associated with poor glycemic control including cardiovascular and perivascular disease, nephropathy, retinopathy, and neuropathy [18].

Many therapeutic approaches using immunological interventions have been investigated in order to prevent or delay the development of T1D. Many clinical trials have been conducted to preserve beta-cell functions by inducing immune tolerance through induction of antigen-specific tolerance or immune suppression. The most commonly used diabetes-specific antigens are insulin and L-glutamic acid decarboxylase (GAD), and these antigen-based immunotherapies are beneficial in a way that they are specific for T1D autoantigens. The immune suppression is usually performed by targeting T cells with anti-CD3 antibodies (i.e. otelixizumab and teplizumab), targeting B cells with anti-CD20 antibodies (i.e. rituximab), and preventing immune activation with the presence of a costimulation blocking agent (i.e. abatacept). Although some trials showed a transient effect, unfortunately, to date no trail have known to succeed the preservation of beta-cell function. There are many other attempts at immune intervention in T1D

as well, yet with mixed results [2, 19]. These unpromising outcomes from previous trails may be related to the choice of antigen or dosing regimen, yet they may also imply that single target immunotherapy trials alone are not sufficient in comprehensively controlling beta-cell destruction. Thus, a consensus is emerging that more effective therapy would be a combinatory therapeutic approach using agents with complementary effects.

In the past, trials targeting more than one molecule at the same time have been tested as multiple targetable pathways are involved in the development of T1D. For example, one of the earliest trials combined mycophenolate mofetil (MMF) with daclizumab (DZB), an immunosuppressant used for organ transplant able to control proliferation of T- and B-cells and an antagonist of α subunit (CD25) of IL-2 receptor on activated T- and B-cells, respectively. A phase one trail focused on combining rapamycin with IL-2, aiming to improve Treg function in recent-onset T1D. Rapamycin, also used in organ transplant, has inhibitory function on pro-inflammatory Th1 and Th17 T cells, and IL-2 works on different cell types expressing IL-2 receptors, stimulating and expanding regulatory T cell populations. More recently, GAD65 loaded aluminum hydroxide vaccine (GAD-alum) combined with vitamin D via intralymphatic and oral administration respectively was tested. Although these trials showed positive results in either delaying disease onset in animal model or increasing Treg cell population in human trails, adverse effects (i.e. neutropenia and leukopenia) were frequently reported, which could be possibly due to that combining molecules at same time would also amplifying adverse effects [20, 21]. This indicates that simply applying an immunosuppressive agent and an immunoregulatory agent is not an ideal approach for developing combination immunotherapy. Learning from all previous research trails as well as appreciating the complex immunological basis of self-tolerance and environmental influences on T1D, we argue that an ideal combined

immunotherapeutic approach should contain a source of diabetes-related antigen that can confer specificity to the treatment, combined with an immunomodulatory agent regulating adaptive immune cells and perhaps favoring the accumulation of Tregs and an anti-inflammatory agent targeting innate immune cells by controlling the release and effect of pro-inflammatory cytokines.

- **Costimulation Blockade (CoB)**

Learning from previous immunological approaches, we attempt to restore regulation of diabetogenic T cells employing a modification of therapeutic strategy called "Costimulation Blockade (CoB)", which controls T cell activation and promotes induction of T cell tolerance by blocking the CD28 pathway. Despite the existence of many other costimulatory pathways, CD28 pathways is one the most potent and well-characterized. The CD28 pathway, which involves CD28 (T-cell-specific surface glycoprotein CD28), CD80 (T lymphocyte activation antigen CD80), and CD86 (T lymphocyte activation antigen CD86), has been well studied, and it playes a pivotal stimulatory role in T cell activation and proliferation. CD28 is a co-stimulator constitutively expressed on the surface of T cells, which binds to CD80 and CD86 expressed on APC surface, and the interaction of CD28 with CD80/CD86 contributes to T cell proliferation via several mechanisms, including increasing IL-2 transcription and mRNA stability, increasing anti-apoptotic protein Bcl-X_L, and decreasing T cell activation threshold [22, 23]. Therapeutic blockade of this pathway in autoimmunity and transplantation models was studied decades ago, which mainly utilized an immunoglobulin (Ig) fusion protein CTLA4-Ig ("abatacept") to bind CD80 and CD86 and thereby blocking the activation of CD28 signaling [22]. Clinical trials using abatacept in autoimmune diseases has long been investigated, and abatacept has been approved

for treatment of rheumatoid arthritis (RA) by the FDA [24]. In type 1 diabetes, abatacept slows the loss of beta-cell function, however, this only applies to the period immediately after diagnosis. Long-term, the inhibition of naïve T cell activation and expansion is limited, and it has no significant differences with placebo [25]. In the context of transplantation, costimulation blockade can help induce immune tolerance of the graft. Research over the past decades has shown that CTLA4-Ig is a potent modulator of transplant rejection and it can induce prolonged graft survival. CTLA4-Ig administrated at the time of graft implantation can induce long-term cardiac allografts in rats, and it can also increase allograft survival of liver, kidney, and lung transplants in rodent animals [26, 27]. In addition to small animals, blockade of CD28 pathway is also capable in inducing prolonged allograft survival in large animals. In CTLA4-Ig treated non-human primates, allogeneic kidney or islet transplants are retained much longer comparing to untreated controls, yet long-term graft survival is not achieved [28].

To date, many findings confirmed the role of CD28 pathway in regulating immune response and blockade of the pathway with CTLA4-Ig is capable of inducing protective effect in both autoimmune and transplant models. In all these models, it is thought that the protective effect of CoB is obtained by promoting an anergic state in T cells and stimulating the accumulation of Treg. However, the effects of CoB by blocking CD28 pathway with CTLA4-Ig alone is still very limited, and itself is not able to prevent all T cell proliferation or transplant rejection, and is not sufficient in inducing tolerance. Many factors have been considered to contribute to the limitation of CTLA4-Ig, including for instance, memory T cell resistance, deleterious effect on regulatory T cells, and non-redundant co-stimulation [29]. In term of transplantation, based on growing body of evidence, the limitation of CTLA4-Ig in inducing long-term graft survival is measurably due to inflammatory responses, which could be caused by infections either pre- or

post-transplantation [30]. These infections are generally caused by pathogenic bacteria, fungi, or viruses and are observed to be associated with acute or chronic transplant rejection. It is suggested that the inflammatory effect is due to the production of pro-inflammatory cytokines by directly response to pathogen and/or to autoantigens (cryptic antigens) exposed following cell damage [31]. The important role of inflammatory cytokines in promoting T cell activation has been introduced previously. Under inflammatory conditions, infections can enhance the maturation of APCs, which lead to increased release of inflammatory cytokines, ultimately promoting the activation of T cells in a CD28-independent manner [30]. In type 1 diabetes, inflammatory responses can be induced via innate signal and/or viral infection, and this might contribute to some understanding of the limited success in long-term preservation of beta-cell function in recent trials. Therefore, we pose to introduce an anti-inflammatory agent into the previously developed "Costimulation Blockade" strategies to inhibit the signaling of inflammatory cytokines and consolidate the modulatory effect of CTLA4-Ig. This will achieve what we define "**Enhanced Costimulation Blockade**" (ECoB), which favors the induction of insulin-specific T cell tolerance and promotion of Treg activity.

- **Enhanced Costimulation Blokade (ECoB)**.

Our previous studies using transplant models have confirmed that the protective effect CTLA4-Ig has on T cells can be neutralized by the inflammatory cytokines released from matured APCs. We were also able to identify a small molecule, **Tofacitinib (Tofa)**, as an anti-inflammatory agent, to provide pharmacological inhibition on the signaling pathway and production of a class of inflammatory cytokines [32].

The role inflammatory cytokines (i.e. Type 1 IFN) played on immunopathogenesis of autoimmune disease, particularly in T1D, has been described previously, and it's been recognized that many cytokines involved in inflammation and autoimmune disease use Jak pathway for intracellular signal transduction [33]. With the existence of two major classes of cytokine receptors, type I and type II, multiple families of cytokines are able to be recognized by this pathway, including interleukins colony stimulating factors (by type I) and interferons and IL-10 related cytokines (by type II) [32]. Upon interaction with their cognate cytokines, cytokine receptors are activated and bind to their paired members of Janus Kinases (Jaks), which includes Jak 1, Jak2, Jak3, and Tyk2. Ligand-induced heterodimerization results in recruiting of multiple signaling molecules (i.e. signal transducer and activator of transcription (STAT)), thus lead to a series downstream signaling of various cytokines that are critical to inflammatory responses [5, 33, 34]. Tofacitinib (Tofa) is a Jak inhibitor that inhibits mainly Jak1 and Jak3. It also has some activity on Jak2, yet negligible on Tyk2 [35]. Mechanistically, Tofa is able to block common γc cytokines signaling (i.e. IL-2, IL-4, IL-7…) through Jak3, and gp130 family (i.e. IL-6 and IL-11), IFN family (i.e. IFN-α and IFN-γ), and IL-10 family (i.e. IL-10) cytokines through Jak1 [32]. Due to the broad range of cytokines Tofa can target, it has been tested as a potential treatment in both transplantation and autoimmune disease. In transplantation, murine cardiac model and nonhuman primate renal model have demonstrated prolonged graft survival when treated with Tofacitinib [36], and clinical trials are currently ongoing to identify the proper dosage of Tofa usage in kidney transplantation [37]. More applications of Tofacitinib are in autoimmune diseases, and it has shown promising results in multiple clinical trials. One of the most successful one is in rheumatoid arthritis (RA), in which, when applied as monotherapy in a phase III trial, symptoms of active RA was reduced and physical function enhanced [38].

Combined with methotrexate in a phase IIb trail, Tofa treatment also showed efficacious results in RA reported by another study [39]. With all these results showing efficacy in treating RA, Tofacitinib has been approved in 2012 by the US FDA to treat moderate-to-severe RA in adult patients with inadequate response to methotrexate [40].

Our group has previously demonstrated in a heterotopic heart transplant mouse model that the "Enhance Costimulation Blockade" (ECoB) strategy utilizing CTLA4-Ig combined with short-course Tofa administration is able to prevent alloreactive T cell activation and promote graft survival, which is mechanistically caused by accumulation of Treg cells in target tissues and reduced generation of effector T cells (i.e. IFN-γ producing Th1). With the demonstrated promising results of the ECoB strategy in transplantation, we plan to further optimize it and apply it for proper immune modulation in Type 1 diabetes.

Tofacitinib is a synthetic drug feasible for oral administration [40], which comparing to current applied administration route is more attractive and ideal as it is a non-invasive, pain free delivery method favoring patient compliance. However, the therapeutic use of this drug is mainly affected by two limitations, side effects and short half-life. Tofacitinib has been reported to have deleterious side effects associated with prolonged systemic use. Serious infection is one of them and it can be caused by virus, bacteria, and fungi. Multiple clinical trials of Tofa have reported increased incidence of opportunistic infections including tuberculosis (TB) and non-disseminated herpes zoster [32]. Another concerning side effect is the development of post-transplant lymphoproliferative disorder (PTLD). PTLD covers a broad range of disorders from hyperplasia to lymphoma, and its incidence has been reported in a phase IIb trail, in which some patients developed it in a broad range of time point after using Tofa [41, 42]. Other side effects that have been reported include: hypercholesterolemia [43], minor increase in creatinine [32], and

increased risk of cancer [44]. Tofa's short half-life is another limitation for its *in vivo* use. Although this might be a potential advantage in controlling infection, maintaining Tofa at a consistent therapeutic concentration within the body becomes very challenging. The pharmacokinetics of Tofa has been reported, and the mean half-life for 5, 15, and 30mg b.i.d doses are 5.2, 5.2, and 3.7 hours, respectively. The peak plasma concentration can be reached within half to an hour following administration, and the elimination half-life is about 3 hours [40]. In order to overcome the aforementioned limitations in Tofa administration, its half-life need to be prolonged and its application need to be modified in a short-term and localized manner. Thus, we propose to introduce nanoparticle delivery system into our ECoB strategy, aiming to achieve short course use as well as controlled and localized delivery of Tofacitinib.

- **Nanoparticle Delivery System**

Nanoparticles are solid or spherical structures typically size in the range of 100-250 nm, and delivery systems using nanoparticles have been studied over the past decades. The manufacturing of nanoparticles using different materials has been characterized, and their utilization in therapeutic development have been demonstrated. Many researches have been focusing on polymer-derived nanoparticles (i.e. Poly(lactic-co-glycolic acid) (PLGA)), and they have been commonly used in biomedical applications due to multiple attractive properties including biodegradability and biocompatibility, well described formulation and adaptability to various types of drugs, possibility to modify surface properties, possibility of site-specific targeting, etc. [45] However, polymeric nanoparticles' application in therapeutic and pharmaceutic approaches remains challenging, as it has limited drug loading efficacy as well as a drug expulsion potential. Although the mean encapsulation efficiency of polymer nanoparticles

is around 60 to 70% depending on drug types, the drug loading efficiency is generally very poor (around 1%) [46], which is a major disadvantage in designing effective delivery systems. Another important pitfall is the high burst release that is generally seen in polymer nanoparticle designs, and this is probably due to the "nano" nature of particles as size is essential and larger particles tend to have smaller initial burst release [46]. Emerging studies on lipid-based particles have shown various unique properties of this class, which makes them an alternative to polymer nanoparticles in therapeutic applications.

The class of lipid-based nanoparticles can be divided into multiple subsets, including solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). They are nowadays considered better drug delivery platforms, as they retain the attractive properties of polymer nanoparticle while minimizing the associated problems [47]. SLN is the first generation of lipid nanoparticles (LNp), and they are made from physiological solid lipids (i.e. fatty acid and triglycerides) and stabilized by surfactant(s) (i.e. poloxamer and polysorbate), which are generally regarded as safe by the FDA [47, 48]. SLN is featured by physical stability, biocompatibility, tolerability, targeted delivery, and controlled release of broad range of ingredients. However, despite exceeding polymer nanoparticles, its drug loading efficiency (approximately 25-50%) is still insufficient and drug expulsion possibility remains high, which is very likely due the use of highly complexed lipid lattice structures and the crystal imperfections formed [49]. In order to improve the feasible properties and overcome the difficulties in SLN, NLC was introduced and developed in the late 1990s. There are so far 3 types of NLCs, the "imperfect type NLC", the "multiple type NLC", and "amorphous type NLC". The first two types are able to enhance drug loading efficiency and prevent drug expulsion using spatially different lipids (at different ratios), by which space between glycerides' fatty acid chains and

crystal imperfections is increased, allowing more guest molecules accommodation. The highest drug load by now is achieved by mixing solid lipids with liquid lipids (oils) at a certain ratio to form a best controlled nanostructure [50]. Since crystallization is the major cause of current drug expulsion in SLN, NLC is able to avoid crystallization upon cooling by mixing special lipids [51]. In addition of reducing drug loading and expulsion problems, lipid-based nanoparticles have been demonstrated by many studies to bypass biological barriers and accumulate in lymphatic system [48, 52, 53], which is a unique property not shared by other nanoparticles that makes them appealing for lymphatic delivery.

Lymphatic system plays an essential role in transporting extracellular fluid, regulating lipid metabolism, and controlling immune responses. In term of transportation, it not only provides channels for lymphocytes and APCs to travel from tissues to draining lymph nodes, but also helps transport dietary lipids from the intestine to the general circulation and helps clear fluid and multiple molecules (i.e. proteins and bacteria) into endogenous carriers from peripheral tissues into systemic circulation [54]. Size of a molecule is important to determine through which pathway they will enter the system. For majority of small molecules, since blood flow rate is much higher (> 100-500-fold) comparing to lymph flow, they tend to drain into blood capillaries from interstitial space rather than lymphatics. Yet for macromolecules (i.e. proteins), entry into blood is usually restricted by their size and their access is promoted to the lymphatics. It has been reported that the pathway for lymphatic uptake of therapeutic macromolecules is similar to that of endogenous macromolecules, in which molecules size 10 nm or lower will enter blood capillaries primarily and those larger than 100 nm will also have restricted access to lymphatic due to reduced diffusion and convection from injection site [55]. Only particles size between 10-100 nm can diffuse through interstitium and access lymphatic vessels [55, 56]. Besides size,

factors that influences interstitial fluid pressure and flow can also alter lymphatic transport [54]. In addition to passively entering lymphatics from interstitial tissue, therapeutics can also be taken up by APCs through phagocytosis in the extracellular matrix and then traffic to draining lymph node via afferent lymphatic vessels, in which size and surface charge also play a critical role. Typically, APCs are more likely to take up larger (500-2000 nm) and positively charged molecules, whereas smaller neutral molecules (< 50nm) will drain directly to lymphatics [57, 58].

To reach the ultimate goal of delivering drug via oral administration, drug delivery systems must first pass the intestinal barrier and access to the interstitial space underneath, which is a common limitation for oral delivery since the stability within the harsh environment of gastrointestinal tract and permeability across intestine are generally very low [54]. For nanoparticles to be taken up intestinally, various characteristics need to be taken into consideration such as size, shape, surface charge, and stability (physical and chemical) [59]. Thus, being able to cross biological barriers is a huge advantage of lipid nanoparticles, and it further grants the possibility of accessing to lymphatic system. Lymphatic uptake of orally administrated macromolecules such as therapeutic proteins and nanosized delivery systems has been studied for decades, and it is suggested that upon absorption by intestine, a large proportion are expected to entering intestinal lymphatics [59].

In addition to transportation, lymph nodes also serve as site for immune surveillance and initiate immune responses, which makes the lymphatic uptake of therapeutic agents an important strategy for treating immune-related disease [55]. After entering lymph node, particles may pass through and then leave via the efferent lymph, or be taken up by different cells within lymph nodes and be retained there. Larger particles are more likely to be taken up by subcapsular

macrophages, whereas smaller ones tend to enter the B or T cell zones [60, 61]. Increasing size increases the efficacy of retaining in lymph nodes, yet it also reduces the likelihood of draining from interstitial sites, thus the balance between lymphatic uptake and lymph node retention is pivotal [62, 63]. Since lymphatics have been recognized having key roles in multiple diseases (i.e. cancer [58], immune and inflammatory diseases [64], and metabolic diseases [65]), lymphatic targeted delivery of drug has become a trend in order to improve therapeutic outcomes, and it is more beneficial in a way that it can enhance drug exposure and increase bioavailability [66] especially following oral delivery. All these properties and advantages described makes lipid-based nanoparticle delivery system a promising candidate for therapeutic delivery of our anti-inflammatory agent Tofacitinib, especially towards our ultimate goal of oral administration.

Our nanomaterial group at the Johns Hopkins Applied Physics Laboratory have been working on manufacturing and optimizing different nanoparticles for the delivery of Tofacitinib. Based on preliminary results, polymer nanoparticles generated with poly(lactic-co-glycolic acid) (PLGA) has very limited drug encapsulation efficiency that is less than 5% and solid lipid nanoparticles (SLN) revealed very high toxicity. Thus, we eventually decide to use nanostructured lipid carrier (NLC) as our delivery vehicle (**Figure 1**). By far our nanomaterial group has been able to define the optimized lipid type, lipid to surfactant ratio, and synthesize method to use, and able to manufacture NLCs with

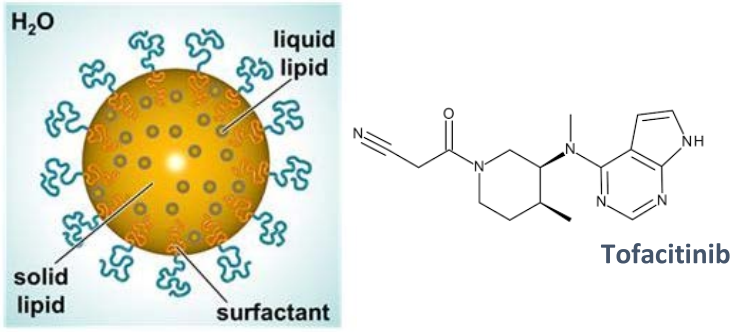


Figure 1. A schematic of NLCs (left) designed to deliver a therapeutic cargo compound, Tofacitinib (right)

characterizations, in term of particle size, polydispersity, and thermal behavior, that are suitable for encapsulating Tofacitinib (Tofa-NLC) with high encapsulation efficacy (79%). To this end, we propose that controlled and localized delivery of Tofacitinib via implementation of biocompatible lipid nanoparticles, Nanostructured Lipid Carrier (NLC), will maximize Tofa's synergy with CTLA4-Ig (actuating ECoB) and promote an effective regulation in the onset and development of type 1 diabetes.

MATERIALS and METHODS

- **Nanoparticles**

All nanoparticles including NLC loaded with far red dye Dilc (Dilc-NLC), NLC loaded with Tofacitinib (Tofa-NLC), and NLC without any cargo (Ctrl. NLC) were manufactured by our collaborators at Johns Hopkins Applied Physics Laboratory. Dilc-NLC was provided at a concentration of 150 mg/ml, in which Dilc was contained at 0.375 mg/ml. Tofa-NLC and Ctrl. NLC were prepared at 150 mg/ml as well, but Tofa-NLC also contained 2 mg/ml Tofa.

- **Animal**

NOD/ShiLtJ (NOD) mice were purchased from The Jackson Laboratory. All mice were housed and bred under barrier conditions in Johns Hopkins University animal facility at Miller Research Building. All animal studies have been proved by Johns Hopkins Animal Care and Use Program.

- **Cell and Tissue Preparation**

Cell Culture. Dendritic cells were cultured from NOD bone marrow in complete medium with growth factors GM-CSF (PeproTech, car#315-03-100UG) and IL-4 (PeproTech, cat#214-14-250UG) at 300U/ ml and 20U/ ml, respectively. Complete medium was made from 500 ml RPMI-1640 Medium (Quality Biological, cat#112-301-101), 50 ml Fetal Bovine Serum (Atlanta, cat#S11095), 10.2 ml Penicillin Streptomycin Solution 50x (Corning, cat#30-001-CI), 5.1 ml Hepes Buffer Solution 100x (Gibco, cat#15630-080), 5.1 ml MEM Non-Essential Amino Acid Solution 100x (Gibco, cat#11140-050), 5.1ml Sodium Pyruvate Solution (100mM) (Sigma, cat#S8636-100ML), 5.1ml Glutamax Solution 100x (Gibco, cat#35050-061), and 500µl beta-mercaptoethanol 100x (Gibco, cat#21986-023). For toxicity, NLC uptake, and maturation tests, bone morrow cells were cultured in 6-well plate ($0.25x10^6$ cells in 2 ml complete medium per plate). For confocal imaging, cells were cultured in p-100 culture dish with $2x10^6$ cells in 10 ml complete medium per plate.

Tissue Digestion. In NLC *ex vivo* and cellular uptake as well as APC maturation test, spleen and lymph nodes were extracted and digested using digestion buffer, which is made from Collagenase IV at 1mg/ml (Worthington, cat#LS004186) and DNase I at 0.1 mg/ml (Roche, cat#10104159001) in RPMI-1640 medium. Digestion buffer was applied 5 ml per spleen and 1 ml per lymph node type, and tissues were digested in incubator for 30 minutes with a magnetic stir bar applied.

- **Flow Cytometry**

Cells were collected after preparation and blocked with anti-CD16/32 (2.4G2, BD Biosciecne, cat#553142), followed by staining of appropriate antibodies against surface proteins in MACs

buffer for 30 minutes at 4ºC. In some experiments, viability dye was stained at a concentration of 0.4 µl/ml in PBS for 20 minutes at 4ºC, and intracellular protein was stained for 30 minutes at 4ºC after fixing and permeabilizing cells with Fix & Perm Buffer (Fixation/Perm Diluent (eBioscience, cat#00-5223-56) and Fixation/ Perm Concentrate (eBioscience, cat#00-5123-43) in 3:1 ratio). In tetramer related experiments, dye and antibodies used included: viability (eBioscience, cat#65-0863-14), anti-CD11c (N418, eBioscience, cat#48-0114-82), anti-CD11b (M1/70, eBioscience, cat#48-0112-82), anti-B220 (RA3-6B2, eBioscience, cat#48-0452-82), anti-TER-119 (TER-119, eBioscience, cat#48-5921-82), anti-CD3 (145-2C11, BioLegend, cat#553061), anti-CD4 (GK1.5, BioLegend, cat#100423), and anti-FoxP3 (FJK-16s, eBioscience, cat#45-5773-82). In all other experiments, cells were stained with following dye and Abs: viability (eBioscience, cat#65-0865-14), anti-CD11c (N418, eBioscience, cat#11-0114-85), anti-CD11b (M1/70, eBioscience, cat#45-0112-80), anti-CD3 (17A2, eBioscience, cat#48-0032-82), anti-B220 (RA3-6B2, BioLegend, cat#103208), anti-CD40 (1C10, eBioscience, cat#12-0401-83), anti-CD80 (16-10A1, BioLegend, cat#104714), anti-CD86 (GL1, eBioscience, cat#48-0862-82). After staining, cells were measured on a BD LSRII flow cytomerter (BD Bioscience) and analyzed using FlowJo software (version 10.5.3, TreeStar)

- **Imaging**

Confocal Imaging. BMDCs were collected and incubated (at $1x10^6$ cells/ml) with Dilc-NLC solution (at 1 µg/ml) in complete medium at 37ºC for different duration. Incubation was stopped by adding ice cold PBS and cells were then stained for CD11c-BV421 (N418, BD Bioscience, cat#565451) in MACs buffer for 20 minutes at 4ºC. Cells were resuspended into MACs buffer at $2.5x10^6$ cells/ml after staining, and 50 µl of the cell solution was added to the center of 35 mm

glass bottom dish (Cellvis, cat#D35-20-1.5-N). Confocal fluorescent images were obtained by ACCM Leica SP8 Confocal Microscope with the utilization of lasers at 405 nm and 638 nm and detectors HyD1 and HyD3. Images were analyzed using the software Leica Application Suite X (LAS X, version 3.5.2).

IVIS. Fluorescent signal in NLC biodistribution and kinetics studies was detected by IVIS Spectrum imaging system (Perkin Elmer), during which the excitation and emission wavelengths were set to 675 and 760nm respectively, and the exposure time set to auto. Average radiant efficiency $(p/s/cm^2/sr)/(\mu W/cm^2)$ in the regions of interest (ROI) was measured as an index to assess the intensity of fluorescent signal.

- **Tetramer Staining**

PE- and APC-labeled $InsB_{10\text{-}23r3}$:I-A^{g7} tetramers were purchased from the National Institutes of Health tetramer core. Cells were collected after preparation and tetramer-binding cells were enriched by staining both tetramers at 6µg/ml in Fc block (anti-cd16/32 (1:100) + Macs buffer + 2% mouse serum + 2% rate serum) at room temperature for exactly 1 hour in dark. Anti-PE (Miltenyi Biotec, cat#130-048-801) and anti-APC (Miltenyi Biotec, cat#130-090-855) MicroBeads were then applied at the volume of the cell pellet for 30 minutes at 4°C, followed by positive selection using autoMacs separator (Miltenyi Biotec). Tetramer-binding cells were collected from the positive column and then underwent flow staining for different markers.

- **Statistical Analysis**

Survival benefit was determined by Log-rank test using Prism software package (version 7.0a, GraphPad Software, 2016). P values less than 0.05 was set to be the threshold for any statistically significant differences.

RESULTS

- **Nanostructured Lipid Carrier (NLC) characterization: Uptake, Biodistribution, and Kinetics**

NLC Toxicity. The toxicity of our formulation of NLC was first tested before any further experiments to assure the particle itself will not harm cells and have a proper working range. The NLC toxicity was assessed by evaluating the induction of cell death following co-culture with NOD bone marrow derived dendritic cells (BMDCs). Titration of NLCs were co-cultured with BMDCs overnight. Cells were then stained for viability dye and dendritic cell surface marker (CD11c) and analyzed via flow cytometry to determine the percentage of dead cells (**Figure 2**). Overall, no significant toxicity was identified at the concentrations tested. NLC particles has low toxicity to DCs until reaching a concentration of 500 μg/ml, where little toxicity starts to be observed, and it guaranteed a relatively broad range of dosage to be applied in the future.

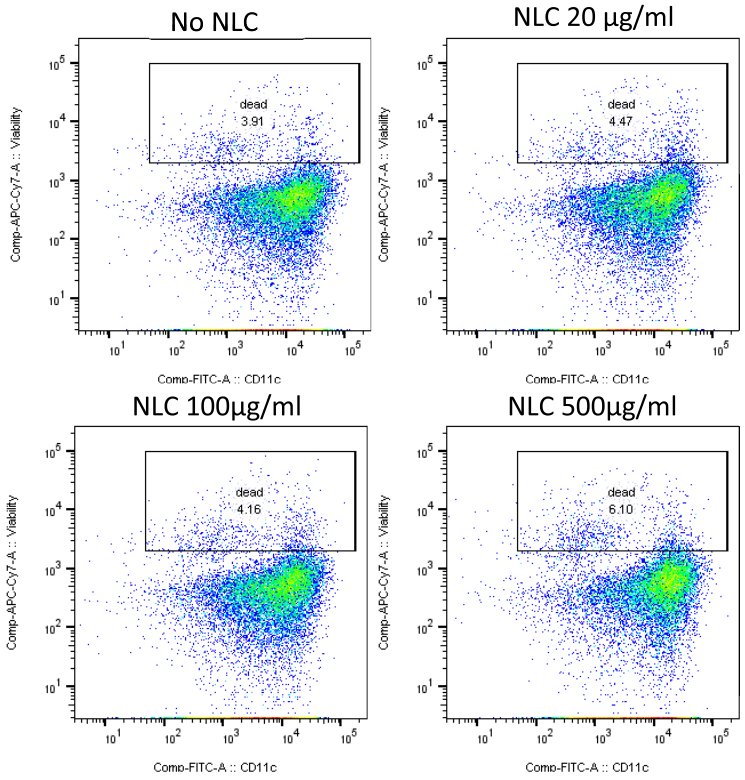


Figure 2. NLC has low Toxicity. Representative results of the assessment of NLC toxicity to NOD BMDCs. Titration of NLC particles (500, 100, 20, or 0 µg/ml) were co-cultured with BMDCs overnight and then stained for viability dye and CD11c antibody to identify viability and dendritic cells. The toxicity of NLC is determined based on the percentage of dead cells, which is gated on viability high cell population within the CD11c+ single cells.

***In Vitro* NLC Uptake**. To study the behavior of our formulation of NLC particles within biological system, how the particle interact with cells and deliver their cargo in particular, we first studied if they can be readily taken up by dendritic cells, an important APC sub-population that plays an essential role in antigen presentation and is also one of the key targets in drug delivery in our design. To study particle uptake as well as their potential uptake profile, fluorescently labeled NLC (Dilc-NLC) containing the far-red dye 1,1',3,3,3',3'-Hexamethylindodicarbocyanine Iodide were co-cultured with BMDCs at different concentration for different amounts of time. NLC uptake was determined by assessing the fluorescence intensity in exposed dendritic cells, and the result (**Figure 3**) shows that at 0.1 µg/ml the uptake of particle is very limited although slight uptake over time can be observed. The signal observed after applying Dilc-NLC at a concentration of 30 µg/ml was overly strong, yet it reveals that our

particle can be taken up by cells. The much stronger uptake was displayed only at initial time frame, yet no further uptake was observed (according to overlapping peaks). Although with limited time points acquired, this indicated that at 10 minutes or even earlier after incubating with DCs at this concentration, the uptake has already reached to a saturation stage. Based on this set of data, 1 µg/ml is the best concentration to apply Dilc-NLC among all concentrations tested, for it's able to show a continuous uptake of the particles over a broad range of incubation time points. Moreover, it reflects that the particles are taken up with an initial quick uptake followed by a continuous gradual one.

With these results shown, our NLC particles are readily taken up by dendritic cells. However, this assessment via flow cytometry does not necessarily reflect whether the NLC particles are attached on the surface of DCs or taken intracellularly. To further explore NLC uptake, confocal microscopy was used to directly and visually study the uptake profile of NLC.

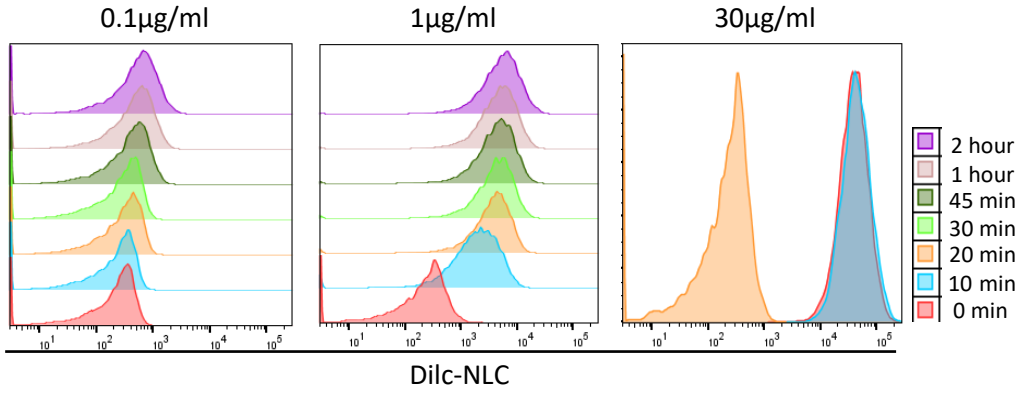


Figure 3.NLC can be readily taken up *in vitro*. Representative cellular uptake of NLC loaded with far-red dye Dilc (Dilc-NLC) by BMDCs. Dilc-NLC at different concentration (0.1, 1, or 30 µg/ml) were co-cultured with BMDCs ($5x10^5$ cells) for different amount of time (0,10, 20, 30, 45, 60, or 120 minutes) in the incubator. Following incubation, cells were stained for dendritic cell marker CD11c. The uptake of Dilc-NLC particle is determined by assessing the fluorescent intensity of the far-red dye via flow cytometry, which is analyzed within CD11c+ single cell population. (Only 3 time points were showed in the 30 µg/ml group due to restrictions experienced at acquisition).

Bone marrow derived dendritic cells were co-cultured with Dilc-NLC at 1 µg/ml (as previously demonstrated) for different amount of time and then underwent confocal imaging for

more precise analyzation. From the confocal images of different exposure time (**Figure 4**), only the contour of dendritic cells is exhibited when no NLC particles were added in, yet NLCs can be clearly seen located inside the plasma area of dendritic cells at all time points when applied. The amount of NLC intake on a per cell base, however, do not seem to differ between initial and later timepoints, and particles can already be observed intracellularly as early as 10 minutes, which all correlate to the flow data represented previously. Using confocal imaging allow us to identify that our formulation of NLC can be readily taken up intracellularly, and it also allows us to study on the uptake process of NLC particles by dendritic cells (experiment ongoing).

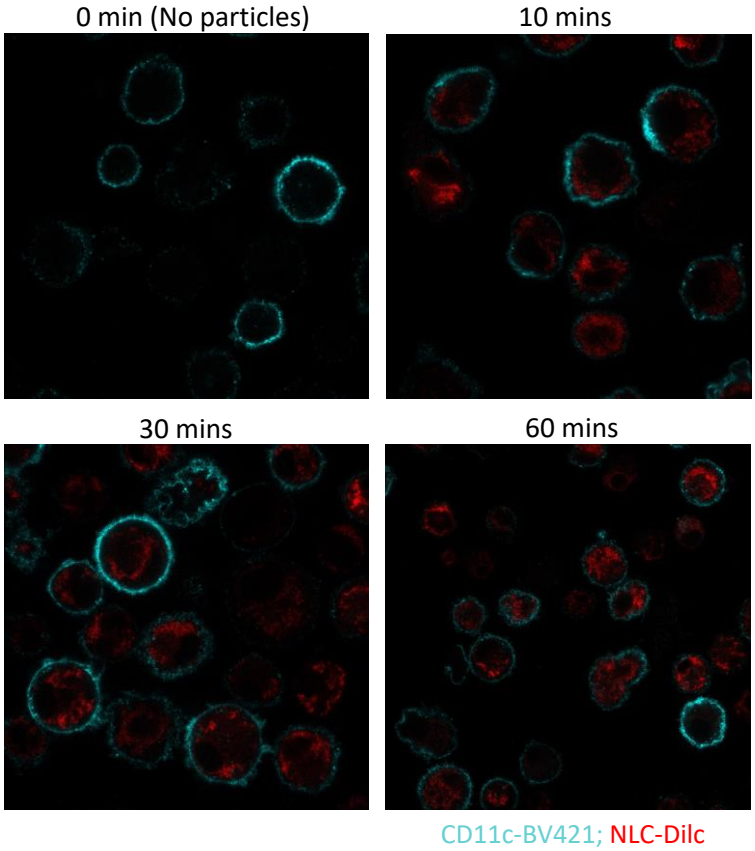


Figure 4.NLC are readily internalized. Representative cellular uptake of Dilc-NLC by BMDCs. Dilc-NLC were co-cultured with BMDCs (5 x10^5 cells) at 1 µg/ml for 0, 10, 30, or 60 minutes, following with surface staining of dendritic cell markers CD11c-BV421. The outline of dendritic cells is represented by CD11c marker (cyan) in all images and NLC particles are represented by Dilc dye (red).

***Ex Vivo* NLC Uptake**. Antigen presenting cell populations are critical for antigen presentation during immune responses and in addition to DCs, they include macrophages and B cells. Considering the much different environment *in vivo*, which lead to the development of

morphologically different DCs and opportunities to encounter various other cell types, we then studied the NLC uptake *ex vivo*, trying to identify their interaction with different cell populations within the lymphatics. Spleen of a NOD mouse was used as it's an important lymphoid organ, and spelenocytes were generated and incubated with Dilc-NLC for different amount of time. Different cell populations within the spleen were identified, including T cells, B cell, conventional dendritic cells (cDCs), monocyte derived dendrtic cells (moDCs), monocytes, and macrophages (**Figure 5a**), and all these cell populations are able to take up NLC particles (**Figure 5b**). The uptake of NLC particles by different cell populations within the spleen confirmed the trend we observed in the previous *in vitro* study: an initial quick uptake of the particle followed by gradual and continuous uptake. Although obtaining similar trend, each cell population exhibited different uptaking profile, implying that the particles are taken up in an active manner.

NLC Biodistribution and Kinetics. Because of complex *in vivo* interactions between particles and internal circulations and tissues, especially with the fact that different administration routes will possibily result in differnet pathways of particle absorption, biodistribution and kinetics of NLC via different administration routes were then studied to better locate particles within the body and identify the optimal time to observe peak uptake. Lipid nanoparticels were often reported to be able to accumulate in lymphatics, thus we started by selecting multiple lymphatic tissues that are involved in the development of T1D to study if our NLC particles also behave in this unique way, and at which time point after administration will the peak uptake occur. To study the impact of differential administration route have on biodistribution and pharmacokinetics *in vivo*, lymphatic tissues including spleen, pancreatic lymph nodes (pLNs),

mesenteric lymph nodes (mLNs), and inguinal lymph nodes (iLNs) were extracted (**Figure 6a**) at different time points after Dilc-NLC administration, and the distribution of Dilc-NLC was determined based on fluorescent intensity employing the In Vivo Imaging Systems (IVIS).

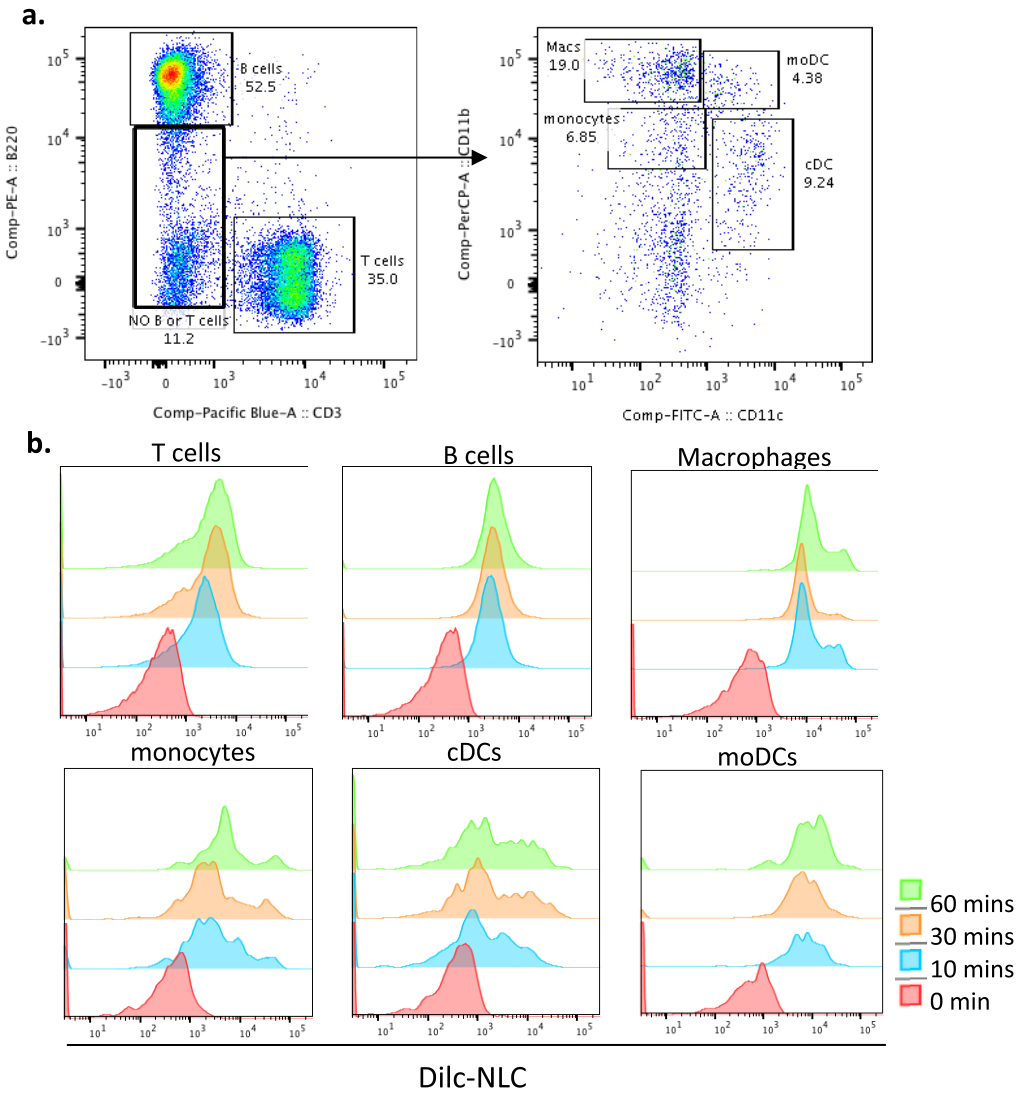


Figure 5. NLC are readily taken up by different immune cell populations in the spleen. Representative cellular uptake of Dilc-NLC by different cell populations. Splenocytes were generated from spleen of a NOD mouse after digestion via single cell suspension, and incubated with Dilc-NLC (1 µg/ml) in incubator for 0, 10, 30 or 60 minutes, following by antibody staining and flow analysis. **a.** Different cell populations within the spleen were identified based on CD3, B220, CD11c, and CD11b expression. T cells were gated on $CD3^{+}B220^{-}$ expression, and B cell on $CD3^{-}B220^{+}$. Non B or T cells ($CD3^{-}B220^{-}$) were further separated into $CD11c^{hi}CD11b^{hi}$ (moDCs), $CD11c^{hi}CD11b^{intermediate}$ (cDCs), $CD11c^{low}CD11b^{hi}$ (macrophages), and $CD11c^{low}CD11b^{intermediate}$ (monocytes). **b.** The uptake of Dilc-NLC is determined through the fluorescent intensity of the far-red dye Dilc, which is analyzed within each cell population identified.

Initially, Dilc-NLC (1 mg) were administrated intravenously (I.V.), intraperitonially (I.P.), subcutaneously (SC), or via oral gavage, and aforementioned lymphatic tissues were extracted 24 hours after Dilc-NLC administration. Mild signals were only observed in spleens (both control and experimental groups) from all routes at this time point, yet their intensity has no big differences with control tissues, and no other tissue from all routes tested showed any signal (**Figure 6b**).

Although particles can be taken up within 10 minutes according to our *in vitro* data, twenty-four-hour checkpoint was originally chosen thinking that much longer time is needed with longer "traveling" and much more barriers to overcome before encountering APCs *in vivo*. However, the particles might be absorbed and eliminated in a much faster manner than expected, and higher dosage of Dilc-NLC might be required to receive stronger signal for any differences to be observed. Therefore, we further reduced the time before tissue extraction and increased the Dilc-NLC dosage. The biodistribution and kinetics of NLCs via oral gavage was first studied as it is the most appealing route and is also our ultimate goal for delivery. The lymphoid tissues were extracted from NOD mice 6 hours after administrating Dilc-NLC (5 mg or 15 mg) via gavage and examined through IVIS. Stronger signal was observed in spleen, pLN, and partially in mLNs of the administrated animals but not in control ones (**Figure 7a**). However, despite that the intensity of signal was positively correlate to dosage, there were no major differences between different tissues in term of average radiant efficiency. Thus, observation time was further reduced to 2 and 4 hours with the more optimal Dilc-NLC dosage (15 mg) applied. We were able to find and extract the other pLN (right) from treated animals this time, and observed NLC accumulation in spleen, one of the pLN (left, same as previous), and most of mLNs, but not in iLNs. Much stronger signal was detected in these tissues, and the strongest appeared at 2 hours

after administration, especially in pLN. At 4 hours, signal already decreased sharply, and it continued to decrease till 6 hours yet in a more gradual way (**Figure 7b**). The highest uptake of NLC seem to occur around 2 hours after administration via oral gavage, and further experiments

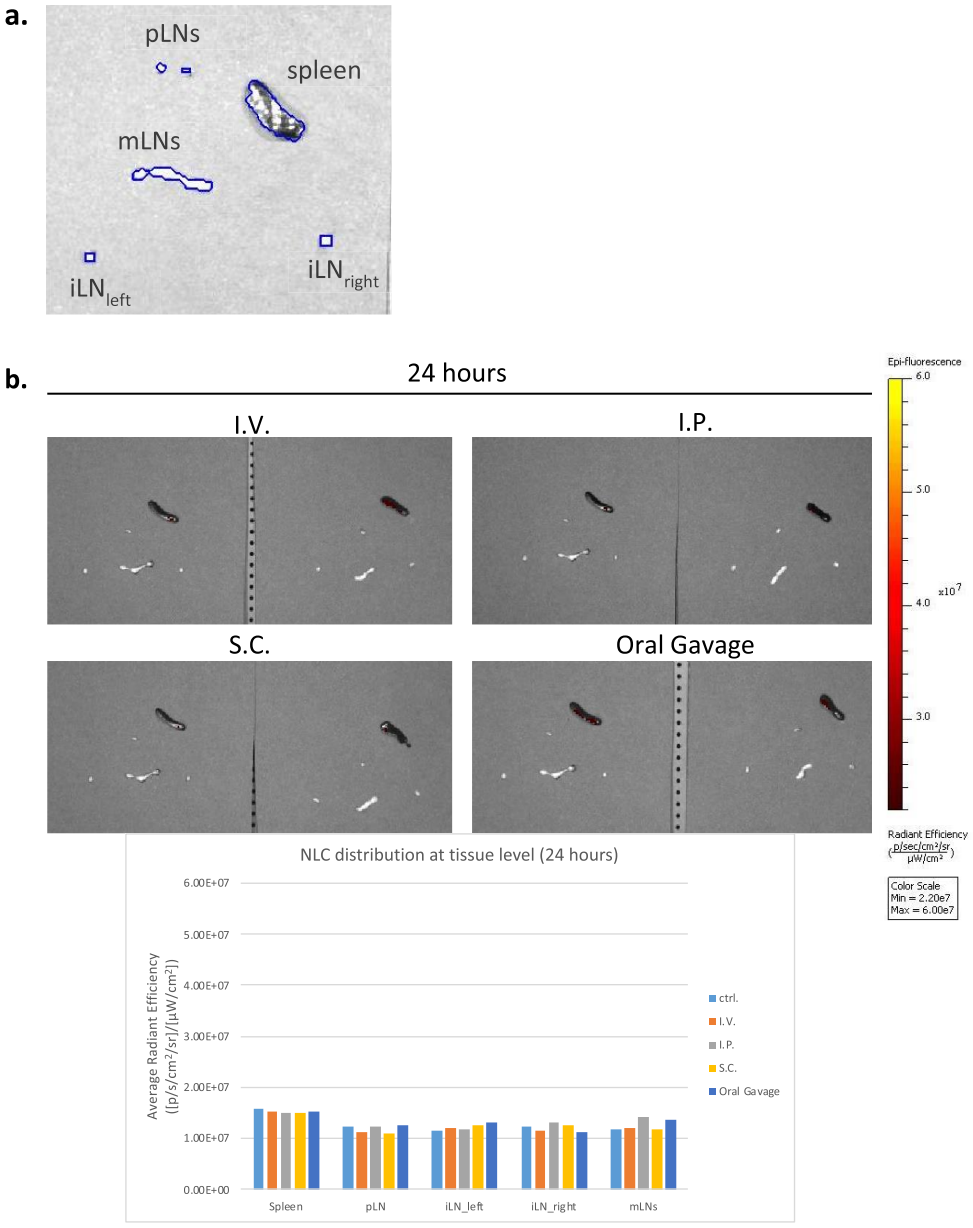


Figure 6. Biodistribution of NLC 24 hours after administration via different routes. Lymphatic tissues of interest including spleen, pLNs, and iLNs were extracted 24 hours after administration of 1 mg Dilc-NLC via I.V., I.P., S.C., or oral gavage. **a**. Relative placement of each tissues during imaging, which also applied to later experiments. **b**. The distribution of NLC after different administration routes was analyzed using IVIS. Tissues from control NOD mouse (untreated) was placed on the left side of each image and treated on the right. Color scale of all images were set from 2.20e7 to 6.00e7. The bar graph shows the intensity of signal of each tissue area.

identified that the peak uptake occurred between 1 to 2 hours after administration (Data not shown). Based on these results, we confirmed that when administrated orally, our formulation of NLC has the unique ability to cross biological barriers and accumulate in the lymphatics, reaching selected tissues: spleen, pLN, mLNs, but no iLNs.

The biodistribution and pharmacokinetics of NLC via I.V. route were then studied using the same IVIS based approach. Considering this route would deliver particles directly into blood stream and circulate around the whole body, which bypasses several physiological barriers, checking timepoint after administration was further reduced as well as the dosage applied. Dosage testing were performed first since large amount of lipid injected into circulation may cause severe health problems (i.e. cerebral thrombosis and pulmonary embolism) and eventually lead to animal death, and 10 mg Dilc-NLC was tested to be able to present strong signal with clear differences between tissues while keeping animals alive till euthanasia (around 2 hours). Dilc-NLC was injected intravenously, and same set of lymphoid tissues were extracted 0.5, 1, or 2 hours after administration. IVIS was used to detect particle distribution, and NLC was found accumulating in all tissues extracted from the Dilc-NLC injected animals, including the other pancreatic lymph node (pLN_{righ}) and both inguinal lymph nodes, at all time points (**Figure 8a**). The signal in tissues of treated animals were much intense comparing to untreated control, and the strongest signal was observed in spleen, which was much than two folds stronger than that in pLN (the one placed on the left) (**Figure 8b**). Comparing to the oral gavage group, more particles (reflected by signal intensity) were able to reach and accumulate to lymphoid sites, and the peak uptake was even earlier around 30 mins to 1 hour (**Figure 8b**). These results indicated that the biodistribution of NLC changed depending on the administration route chosen, with a

related alteration of the peak uptake time as well. The impact that intraperitoneal and subcutaneous routes have on NLC distribution and kinetics are currently being investigated.

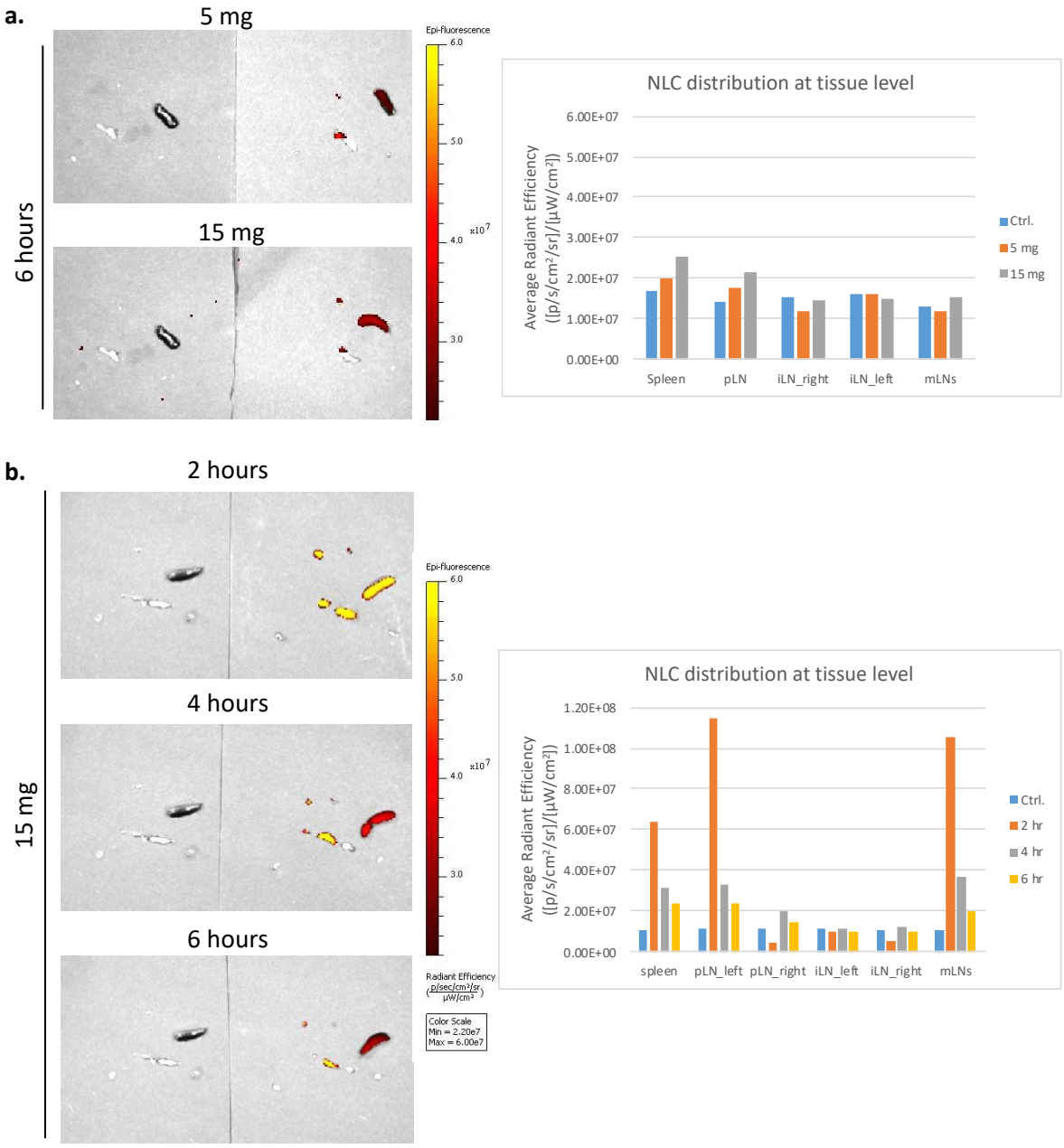


Figure 7. The impact of oral gavage administration route on NLC biodistribution and kinetics. Dilc-NLC was administrated into NOD mice via oral gavage and lymphoid tissues including spleen, pLN(s), mLNs, and iLNs were extracted at different timepoints (untreated at left; Dil-NLC administrated at right). The distribution and uptake of NLCs were determined by fluorescent intensity by IVIS. The color scale is set from 2.2e7 to 6.0e7, and the intensity of fluorescent signal from each tissue is shown on the bar graph. **a**. Dilc-NLC at 5 or 15 mg were given to NOD animals and lymphatic tissues were extracted 6 hours after administration. **b**. Dilc-NLC at 15 mg were given to NOD animals and lymphatic tissues (also including another pLN (right) for treated animals) were extracted 2, 4, or 6 hours after administration.

***In Vivo* NLC Uptake.** Our previous studies have demonstrated that NLC particles are taken up (*in vitro*) by different subsets of APCs and other cell populations. Therefore, it is very important to study whether NLCs can be taken up by different cell populations in vivo as multiple biological barriers exist and need to be overcome before encountering lymphocytes within lymphoid tissues. The biodistribution studies have shown that NLCs can successfully reach and accumulate into lymphoid sites after being delivered intravenously or via oral gavage. We then studied the uptake profile within those tissues to identify which specific cell populations were taking up the particle. The highest uptake of NLCs happens 1 to 2 hours after oral gavage,

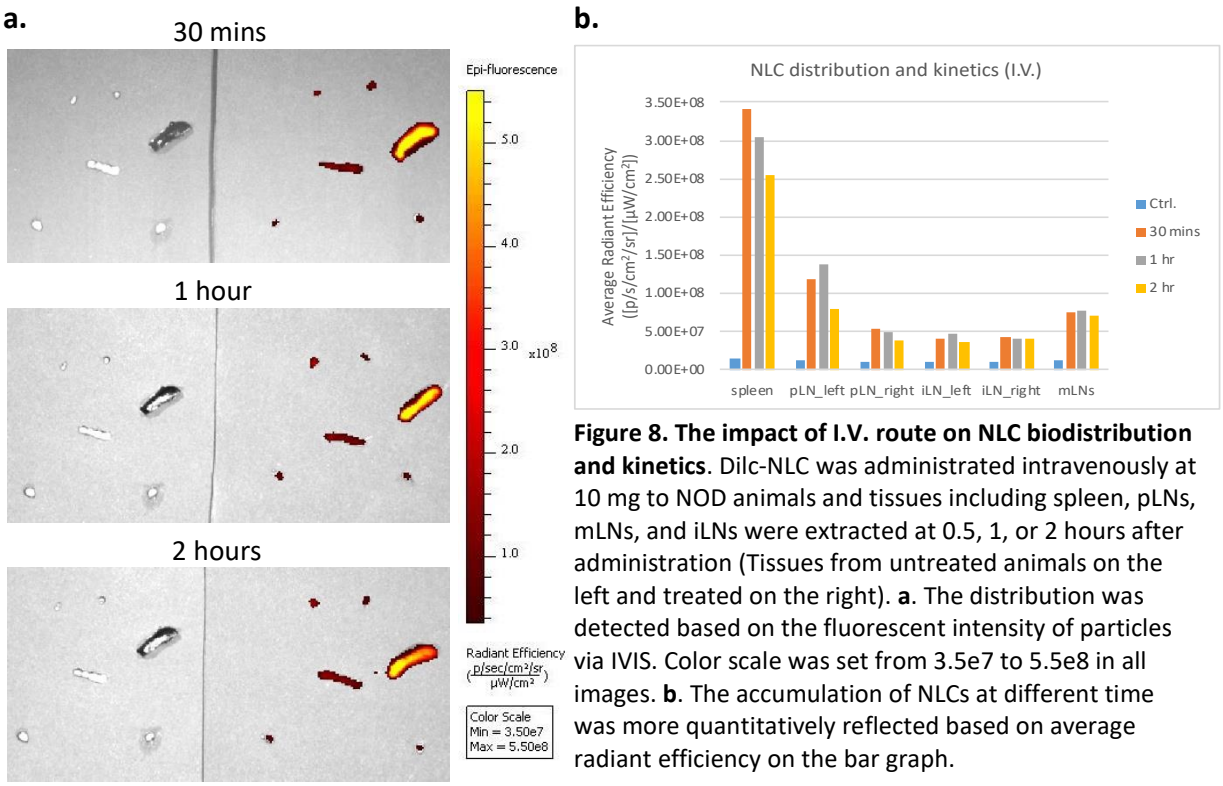
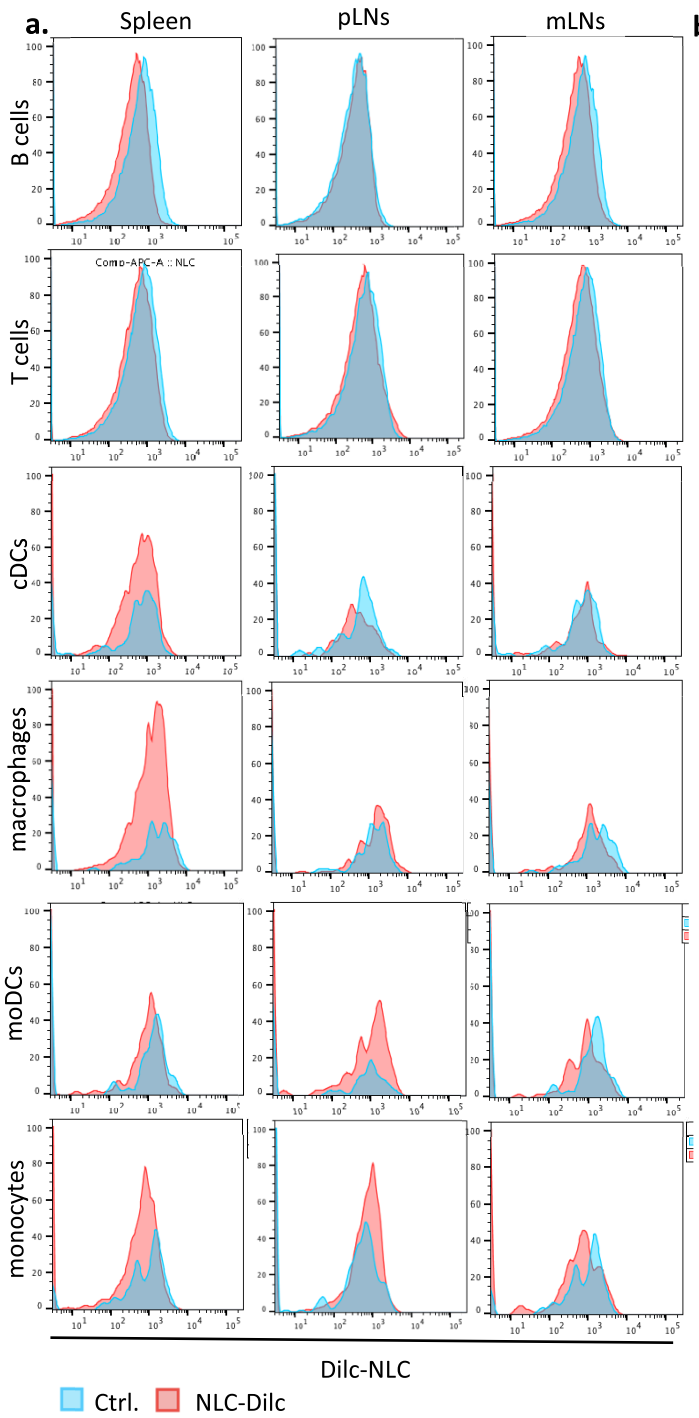


Figure 8. The impact of I.V. route on NLC biodistribution and kinetics. Dilc-NLC was administrated intravenously at 10 mg to NOD animals and tissues including spleen, pLNs, mLNs, and iLNs were extracted at 0.5, 1, or 2 hours after administration (Tissues from untreated animals on the left and treated on the right). **a**. The distribution was detected based on the fluorescent intensity of particles via IVIS. Color scale was set from 3.5e7 to 5.5e8 in all images. **b**. The accumulation of NLCs at different time was more quantitatively reflected based on average radiant efficiency on the bar graph.

thus the lymphoid tissues including spleen, pLNs, mLNs, and iLNs from Dilc-NLC treated animals were extracted 1.5 hours after oral administration. Tissues were digested to separately generate splenocytes and lymphocytes, and the uptake of NLC was determined by assessing the fluorescent intensity detected in different cell populations via flow cytometry. As inguinal lymph

nodes showed fluorescent intensity very close to that of untreated control animals at all timepoints and dosage checked (Figure 7), they were used as control tissue in this experiment. Although the difference in signal intensity between control and the rest lymphoid tissues was clear on IVIS, no significant differences on Dilc intensity were observed among different cell populations within spleen, pLNs, or mLNs (**Figure 9a**), and this result was consistent with 2 more repeats. Thus, although tissue level NLC uptake can be observed via IVIS, cellular uptake of particles cannot be detected via flow cytometry. This might due to limited Dilc intensity within each cell. With much stronger signal intensity detected at each lymphatic tissue on IVIS, cellular uptake of NLC via I.V. were then studied hoping this time fluorescent would exceed the detection threshold of flow cytometer. Spleen of untreated and 10 mg Dilc-NLC treated animal were extracted 30 minutes (as previously demonstrated to be within the highest uptake range) after intravenous administration, and only spleen was extracted here since not enough cells were obtained from other lymphatic tissues after processing. Cellular level uptake of NLC was clearly detected, with major peak shift observed compared to control, in all cell populations identified (B cells, T cells, cDCs, moDCs, macrophages, and monocytes) within the spleen (**Figure 9b**). Therefore, our formulation of NLCs can also be taken up by different populations of splenic cells *in vivo*, however, the cellular uptake can only be detected by flow cytometer when administrate via I.V.



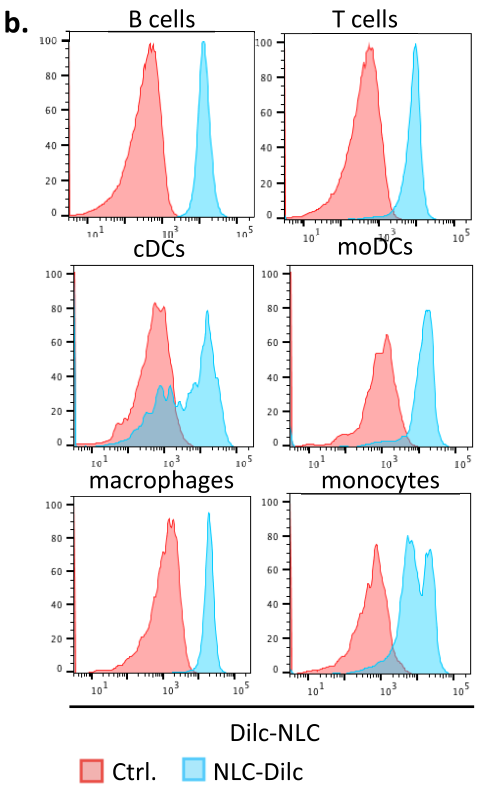


Figure 9. NLC uptake in vivo following oral or intravenous administration. **a**. Representative cellular uptake of NLC 1.5 hours after orally administrating 15 mg Dilc-NLC. Lymphatic tissues including spleen, pLNs, mLNs, and iLNs were extracted from NOD animal. Tissues were digested according to digestion protocol, and splenocytes or lymphocytes were then generated from each tissue via single cell suspension, followed by surface staining of anti-CD3, anti-B220, anti-CD11c, and anti-CD11b antibodies. Different cell populations were identified using the same gating strategy shown in Figure 5a. **b**. Representative cellular uptake of NLC 30 minutes after administrating 10 mg Dilc-NLC intravenously. Spleen was extracted, digested, and splenocytes were generated via single cell suspension followed by surface staining of anti-CD3, anti-B220, anti-CD11c, and anti-CD11b antibodies. Same gating strategy as previous was applied.

- **NLC Mediated Delivery of Tofacitinib**

With all the NLC characterization on uptake and biodistribution, this formulation of particle seems to be promising on delivering the cargo of our interest, tofacitinib (Tofa), as it tends to accumulate at lymphatic tissues and can be taken up by multiple cell populations as well. Thus, we tested whether Tofa encapsulated NLC particles (Tofa-NLC) were capable of delivering bioactive Tofa to our cell populations of interest, particularly DCs. Inspired by previous demonstrations of our group, in which exposure of DCs to soluble Tofa can influence the upregulation of the maturation markers induced by the maturation stimuli LPS, we hereby tested the delivering capacity of Tofa-NLC by measuring their ability to inhibit the maturation of DCs in response to LPS. Bone marrow derived dendritic cells (BMDCs) were exposed to Tofa-NLC

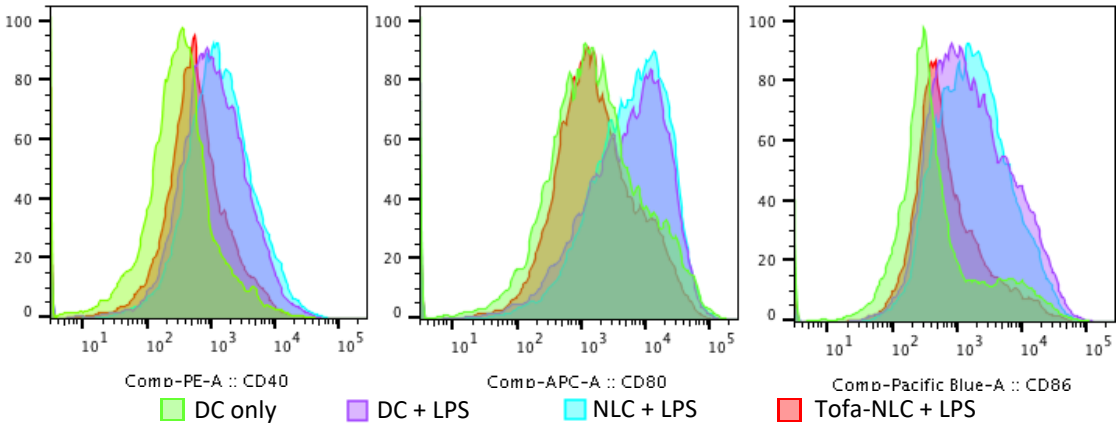


Figure 10. NLC mediated delivery of Tofacitinib. Bone marrow derived dendritic cells (BMDCs) were cultured and exposed to NLC itself (Ctrl. NLC) or NLC loaded with Tofacitinib (Tofa-NLC) for 6 hours (both particles at a final concentration of 30 µg/ml), followed by LPS stimulation at 200ng/ml per condition overnight. Cells were collected and stained with dendritic cell marker CD11c and maturation markers CD40, CD80, and CD86, DC maturation level were then assessed via flow cytometry

or NLC particle itself (Ctrl.NLC) followed by stimulation with LPS, and their maturation level was measured by analyzing the expression of cell surface maturation markers CD40, CD80, and CD86 via flow cytometry. Dendritic cells with no exposure to particles showed upregulation in all markers after stimulation, and those DCs exposed to Ctrl. NLC showed similar maturation

level in term of the expression of maturation markers (**Figure 10**). The upregulation of maturation markers was prevented after exposing to Tofa-NLC, and the maturation was reduced to a level close to that measured in DCs without any stimulation (**Figure 10**). This data clearly shows that our formulation of NLC is able to deliver bioactive anti-inflammatory agent Tofacitinib to DCs, which lead to the inhibition of DC maturation.

- **The Impact of Enhanced Costimulation Blockade (Tofa-NLC + CTLA4-Ig) on T1D**

The most frequent animal model used in researching type 1 diabetes is the non-obese diabetic (NOD) mice, animals that spontaneously develop the disease similarly to humans. In NOD mice, insulitis (infiltration of lymphocytes into pancreatic islets) starts very early at 3-4 weeks of age followed by overt hyperglycemia beginning at age 10-12 weeks, in which 70-75% of functional beta-cells in pancreatic islets have been destroyed. Differences in development of diabetes between male and female NODs are observed, and under specific pathogen-free (SPF) conditions, females develop diabetes much faster than males (around 90% of females are diabetic by 40 weeks, while males only around 30%) [11, 67]. NOD mice were used in studying the impact of our ECoB strategy on T1D, and considering that sex is a variable in the development of the disease, only females were used in these experiments. Treatments were initiated at two stages, either early at 3 weeks old or later at 10 weeks old, since insulitis starts way early than diabetes can be detected. Diabetes was monitored by measuring blood glucose level weekly since week 10 of age, and a NOD is determined to be diabetic when its blood glucose exceeds 250 mg/dL for two consecutive days.

The Impact of Tofa-NLC on T1D Development. Before studying the ECoB strategy, we first studied whether Tofa-NLC itself would has any impact on the development of T1D. Tofa-NLC was administrated to NODs via oral gavage at 3 or 10 weeks of age. Both early and late stage treatments showed therapeutic effects in delaying the onset of diabetes comparing to untreated control animals, especially long-term (> 23 weeks), and the therapeutic effects exhibited in both groups were significantly different ($p<0.05$) (**Figure 11a**). The releasing profile of Tofa-NLC was only recently determined by our collaborators at APL, which showed that approximately 90% of the Tofa loaded in the particles is released in the initial 24 hours. With this information, we are currently testing the impact of Tofa-NLC to 3- or 10-weeks old NODs when delivered via an intense regimen, in which total 5 administrations of Tofa-NLC was given one per day for 5 days via oral gavage, yet it is still too early to determine whether there are any therapeutic effects with this new regimen.

The Impact of CTLA4-Ig and CTLA4-Ig + Tofa-NLC on T1D Development. CTLA4-Ig (abatacept) prolongs graft survival in transplantation, as described by various studies, while dosage remains critical since it can lead to opposite results. Preliminary data show that only CTLA4-Ig treated at later stage (10-week) with a lower dosage (500:250) seem to show some positive result, while higher dosage (500:500) at later stage and both dosages at earlier stage give negative result in delaying diabetes onset comparing to untreated control NODs (Data not shown). Hereby, we treated newly defined high dose (500:250) CTLA4-Ig to 10-week-old NODs, and reduced the dosage according to animal weight to low dose (250:125) for 3-week-old animals. Neither dosage provided protective effect on diabetes onset, and further tested low dose (250:125) CTLA4-Ig applied at later stage did not show protective effect either (**Figure 11b**).

Combined therapy (CTLA4-Ig (500:250) + Tofa-NLC) was also applied to NODs at later stage, yet surprisingly it again had negative effect on the onset of diabetes, if not behaving even worse comparing to CTLA4-Ig applied by itself at the same stage (**Figure 11b**). Therefore, these data suggest that neither CTLA4-Ig by itself or combined with Tofa-NLC at the dosage we tested will provide any therapeutic impact on the development of T1D.

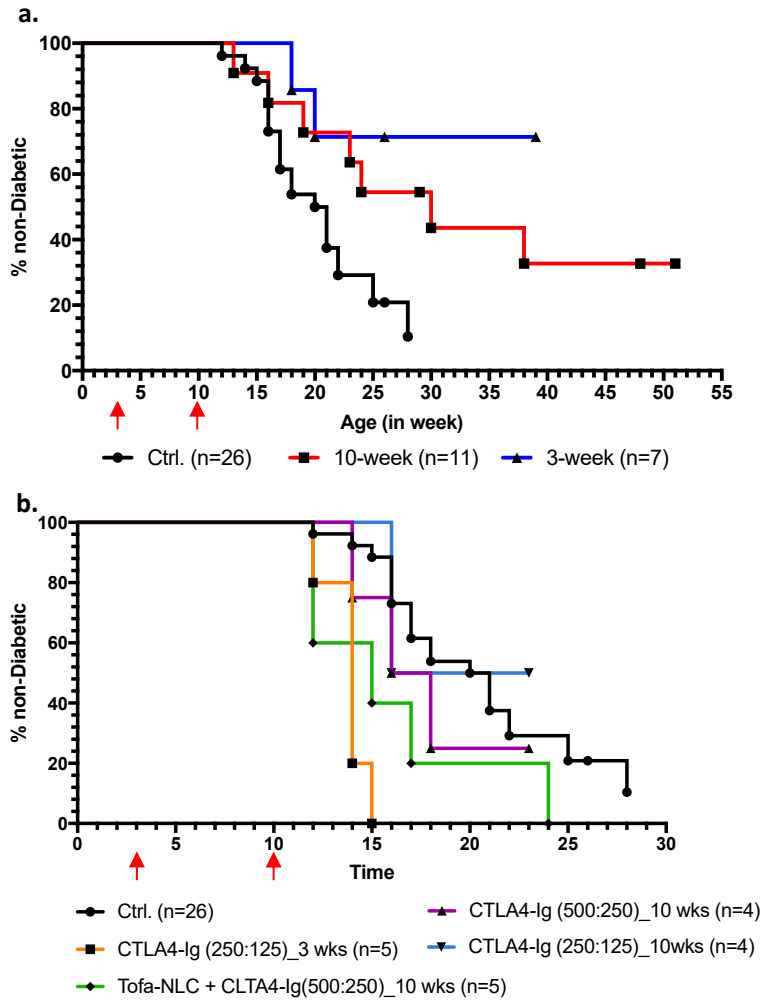


Figure 11. The impact of ECoB on T1D. **a**. Tofa-NLC were administrated to 3- or 10- week-old NODs via oral gavage one administration per day, every other day for 5 administrations. Total of 0.5 mg or 1 mg equivalent Tofa was delivered to 3- or 10- week-old NODs over the treatment range, respectively. Blood glucose levels were monitored weekly since week 10 of age, and diabetes was determined when blood glucose exceed 250 mg/dL for two consecutive days. **b**. CTLA4-Ig alone was adminsitrated to 3- or 10- week-old mice via I.P. for 4 total administrations at day 0, 2, 4, and 6. Either high dose (500: 250) or low dose (250: 125) were applied, in which 500 or 250 μg of CTLA4-Ig was injected at the first administration while 250 or 125 μg was injected in the following ones, respectively. CTLA4-Ig was also used together with Tofa-NLC, during which 4 high dose (500: 250) CTLA4-Ig were injected via I.P. at day 0, 2, 4, and 6, while total of 1 mg Tofa equivalent Tofa-NLC was delivered via oral gavage at day 0, 2, 4, 6, and 8.

Elucidate the Mechanism Behind Therapeutic Effect of Tofa-NLC on T1D. Maturing antigen presenting cells has been shown to closely related to inflammatory cytokines release, and our previous experiments demonstrated that Tofa-NLC can inhibit the maturation of dendritic

cells *in vitro*. Thus, the maturation level of different subsets of APCs within lymphatic tissues were tested after treating Tofa-NLC via oral gavage with regular regimen in order to understand whether this is the reason of the therapeutic effect observed. Maturation level was assessed in multiple tissues (spleen, pLNs, mLNs, and iLNs) by measuring the expression of maturation markers (CD40, CD80, and CD86) on different subsets of APCs (**Figure 12a**), however, no reduction in the expression of maturation markers was identified among APC subsets within different tissues of Tofa-NLC treated mouse compared to untreated control (**Figure 12b**). To further confirm this is not due to limited detection by flow machine, as we previously experienced in studying the cellular uptake of Dilc-NLC *in vivo*, we repeat the same experiment but administrating Tofa-NLC via I.V. There was again no inhibition on APCs' maturation level between the two groups of animals (**Figure 12c**), therefore, the positive effect Tofa-NLC exhibited on delaying the onset of T1D is not a result of inhibiting APC maturation.

Other ongoing experiments are focusing on studying the mechanism related to the protective effect provided by Tofa-NLC. We are examining lymphocytes infiltration in pancreas at different age with or without Tofa-NLC treatment, in which histological sections of pancreas are obtained and insulitis is scored in 10- and 12- week-old, Tofa-NLC treated (at early or later stage) or untreated NOD animals. Given that Treg is critical in controlling the adaptive immune system as well as in preventing autoimmune disease, we are also identifying the proportion of Treg population within insulin specific T cells in 10-week-old NOD mice treated with (at early or later stage) or without Tofa-NLC. Insulin reactive T cells can now be successfully isolated from CD4+ T cell population via InsB specific tetramer staining (**Figure 13a**), and Treg population within insulin reactive T cells can be identified. Treg proportion from 10-week-old NODs receiving Tofa-NLC at early stage have been studied, yet it varies from approximately

12% to almost 40% (**Figure 13b**). Their proportion in untreated or Tofa-NLC treated (at later stage) animals is still underway.

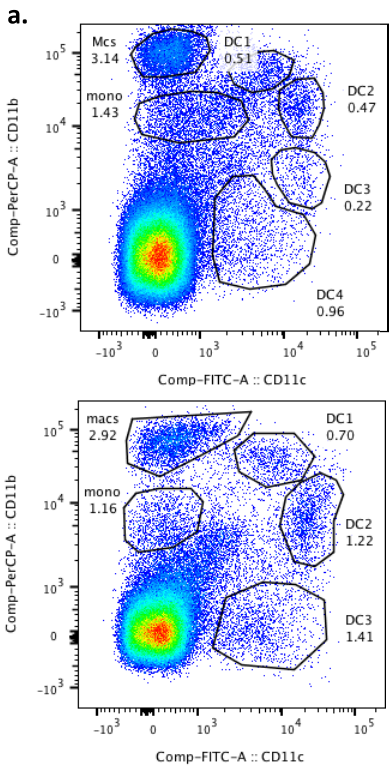
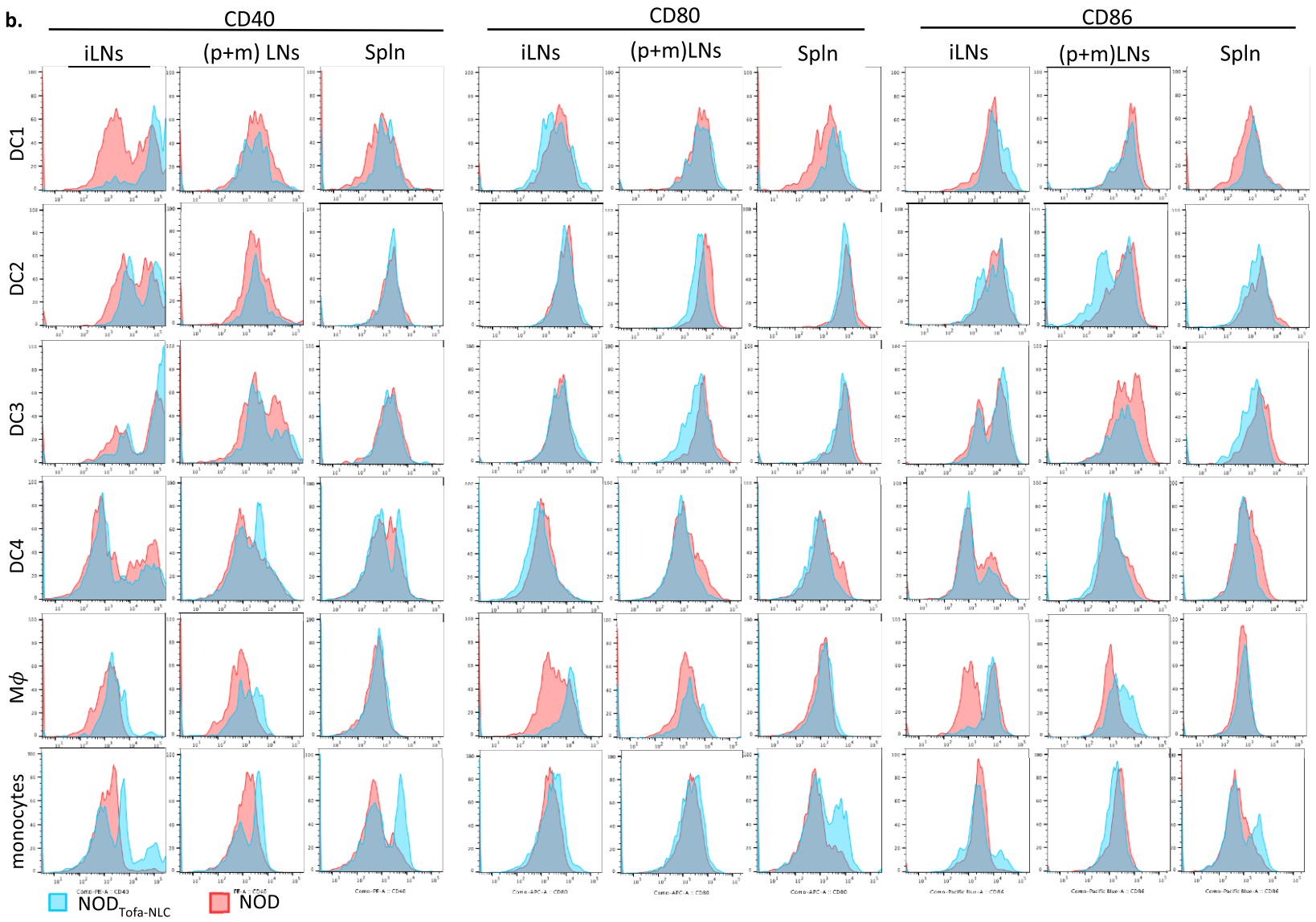


Figure 12. Maturation studies of APCs. NOD animals were treated with or without Tofa-NLC through different routes. The day after last administration, lymphatic tissues including spleen, pLNs, mLNs, and iLNs were extracted and digested. Splenocytes/ lymphocytes were then generated via single cell suspension followed by staining of anti-CD11c, anti-CD11b, anti-CD40, andti-CD80, and anti-CD86 antibodies, and the maturation was determined by measuring the expression of maturation markers via flow cytometry. **a**. Different subsets of APCs can be identified, yet with limited markers, subsets of DCs cannot be precisely identified. (top: from oral gavage; bottom: from I.V.) **b**. Representative maturation markers' expression in APCs of different lymphatic tissues after administrating Tofa-NLC via oral gavage. Tofa-NLC containing total of 1 mg equivalent Tofa was given via oral gavage over 5 administrations every other day for 9 days (day 0-8). **c**. Representative maturation level of APCs after I.V. administration. Tofa-NLC containing total of 0.12 mg equivalent Tofa was injected intravenously over 3 administrations during 3 consecutive days. The control animal here was treated with PBS using the same regimen.


b.
CD40
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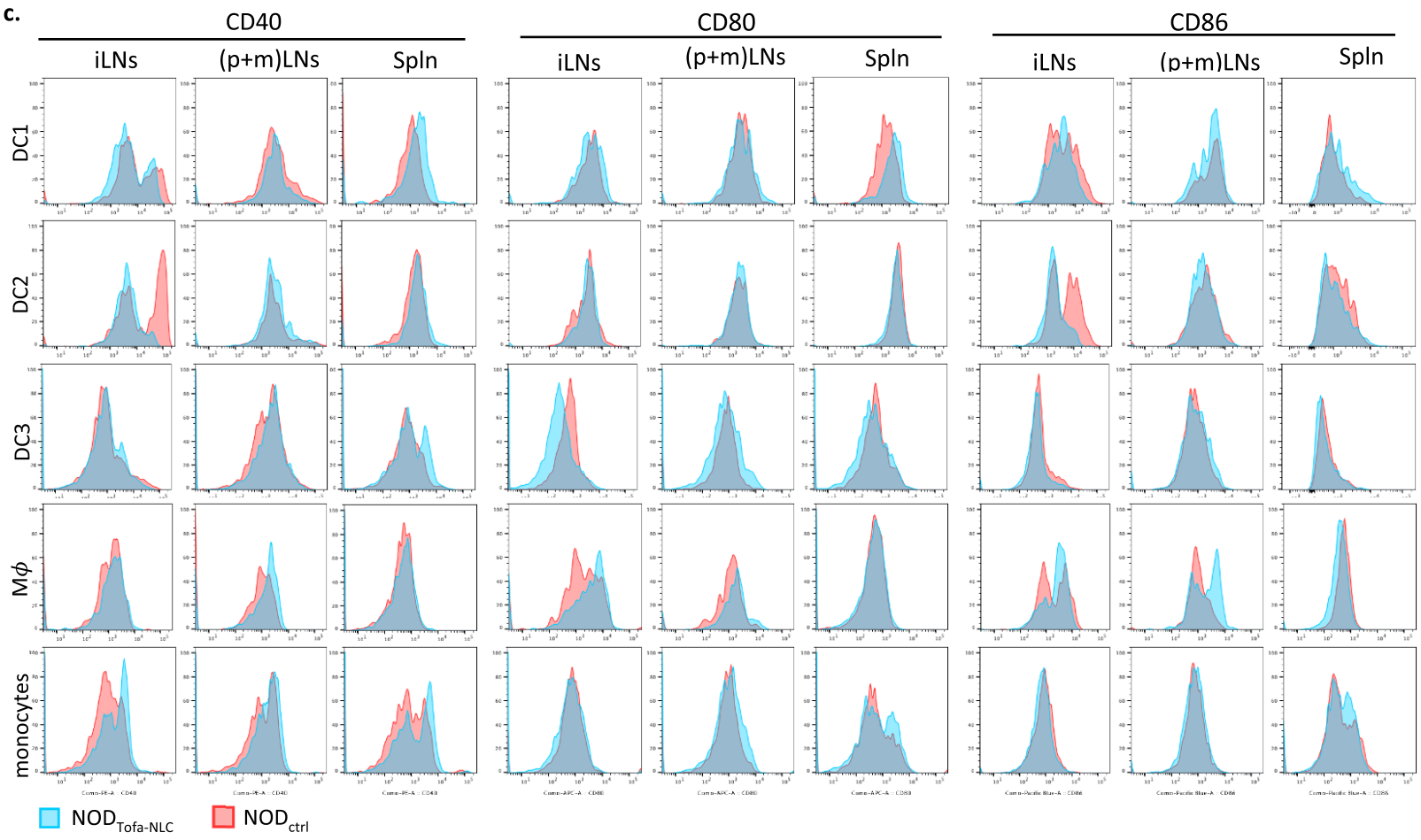
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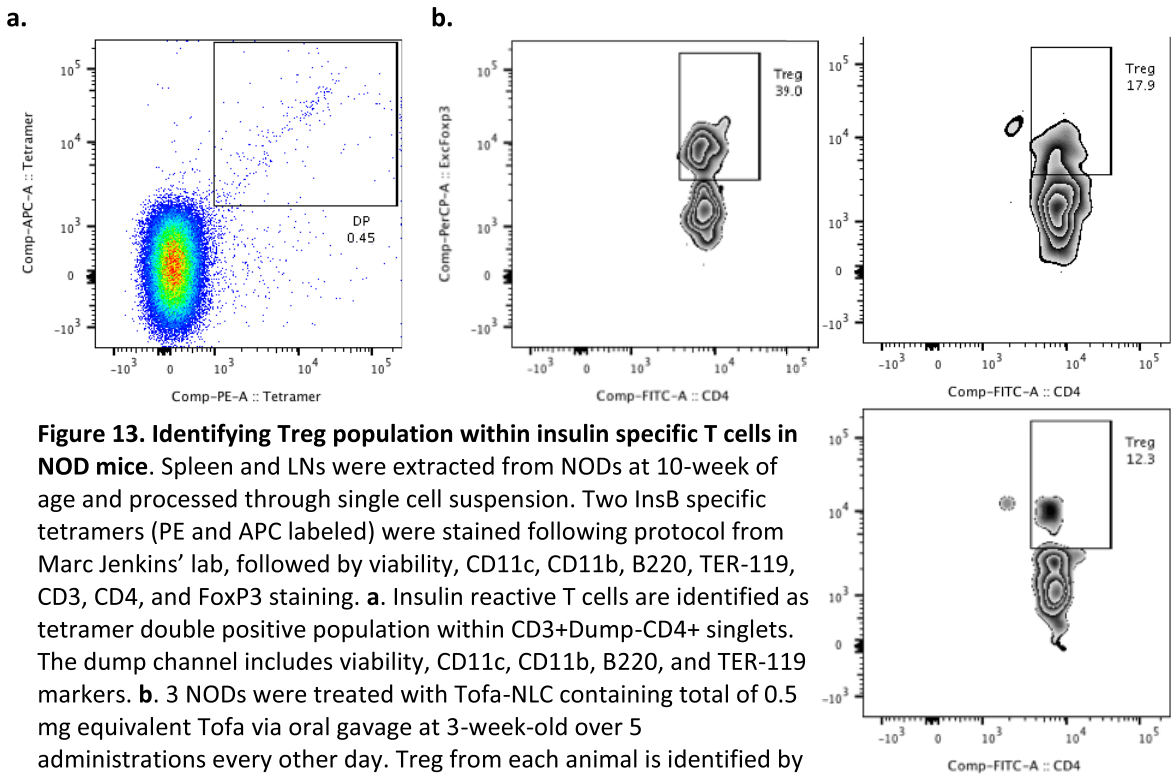


Figure 13. Identifying Treg population within insulin specific T cells in NOD mice. Spleen and LNs were extracted from NODs at 10-week of age and processed through single cell suspension. Two InsB specific tetramers (PE and APC labeled) were stained following protocol from Marc Jenkins' lab, followed by viability, CD11c, CD11b, B220, TER-119, CD3, CD4, and FoxP3 staining. **a**. Insulin reactive T cells are identified as tetramer double positive population within CD3+Dump-CD4+ singlets. The dump channel includes viability, CD11c, CD11b, B220, and TER-119 markers. **b**. 3 NODs were treated with Tofa-NLC containing total of 0.5 mg equivalent Tofa via oral gavage at 3-week-old over 5 administrations every other day. Treg from each animal is identified by FoxP3+, which is gated within tetramer double positive populations.

DISCUSSION

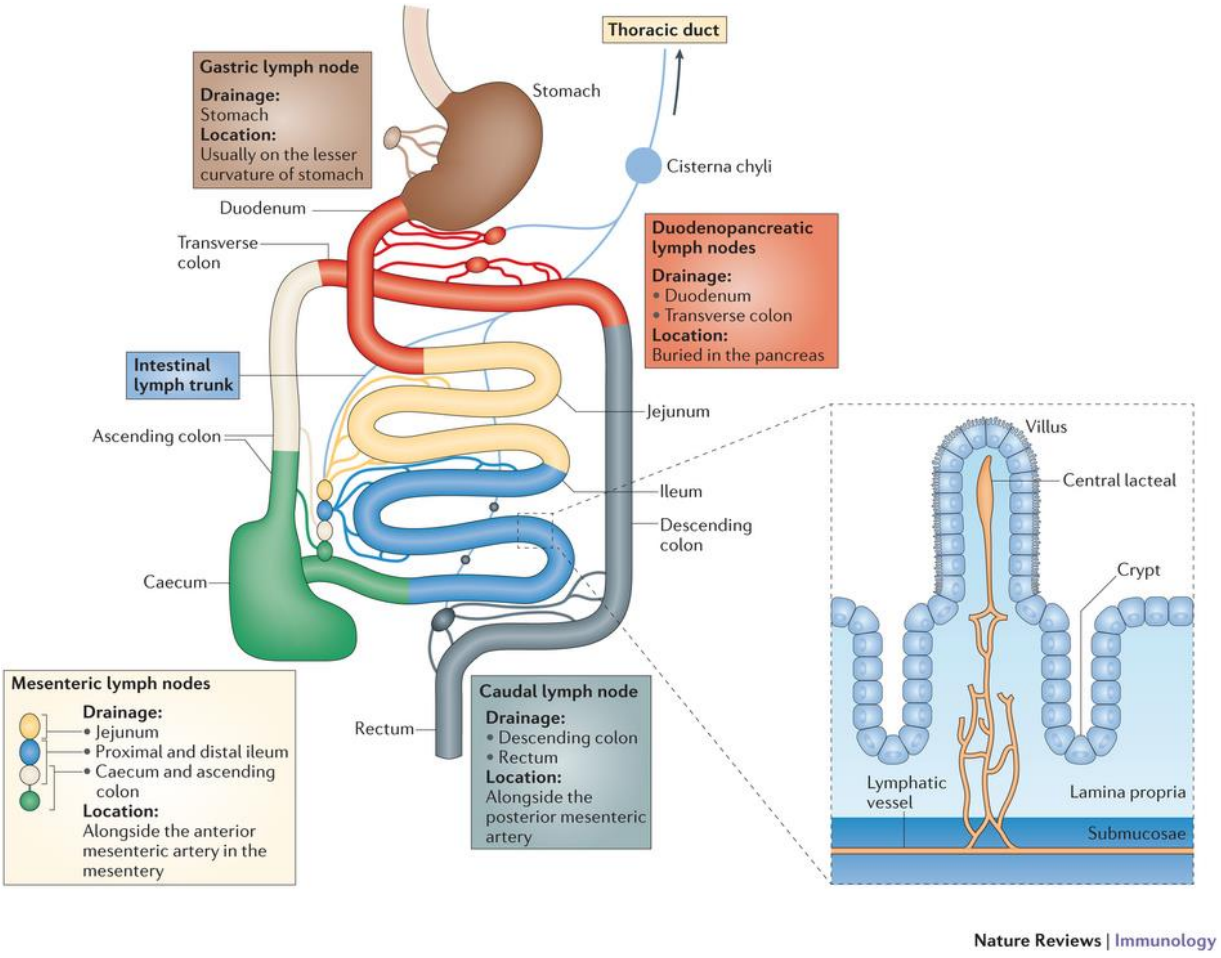
In summary, Our work demonstrated that our formulation of nanostructured lipid carriers (NLC) is readily take-up by different subsets of antigen presenting cells intracellularly, and able to cross biological barriers and accumulate in lymphatic tissues involved in T1D. It is also able to mediate the delivery of bioactive anti-inflammatory agent Tofacitinib (Tofa). The use of Tofa-NLC alone is able to provide therapeutic effect on delaying the onset of T1D when administrated via oral gavage, however, in combined use with CTLA4-Ig or applying CTLA-Ig only did not give any protective effect. Mechanistically, the mechanism behind the therapeutic effect caused by Tofa-NLC treatment is still unclear, yet studies such as determining the regulatory T cell proportion within insulin reactive T cells before and after treatment is currently underway.

Confocal imaging shows NLC can be taken intracellularly, but current investigation does not provide information on the intake process, whether through endocytosis, diffusion, or some other ways. Neither can it tell whether within cytoplasm, the particle remains intact or are being lysed as only the Dilc dye loaded can be overserved through the microscope. Future experiments, such as applying confocal time-lapse, are needed in order to better observe and study the intake process of this formulation of NLC, and a comparison experiment using Dilc dye alone without NLC is necessary to test on particle status within cytoplasm. We also need to keep in mind that confocal microscope detects fluorescence based on pixel, which in our case is 200 nm, yet the size of our NLC particle is approximately 70 nm. Thus, fluorescence detected and observed on those confocal images is probably aggregation of particles rather than individual ones, and the size of the fluorescent dot appeared to correlate to the amount of particle aggregated.

Our NLCs also have the unique property of accumulating into lymphatic tissues, and it is very interesting that signals can only be detected from one of the pancreatic lymph nodes when administrated via oral gavage, but from both pLNs when administrated intravenously. Administration via oral gavage requires active participation of the intestine, which provides absorption and digestion of metabolites, and it has been long recognized that specific lymph nodes drain different intestine segments [68]. Although both pLNs are closely located next to each other, one of them drains primarily the duodenum, while the other drains the transverse colon (**Figure 14**), and this can probably explain why signal was only observed in one pLN after oral delivery of Dilc-NLC. The node that particles mainly accumulated to is likely to be the one draining form duodenum as it's the initial place within intestine encountering NLCs after oral delivery. Within the time of observation, probably only limited particles are able to travel to and absorbed by the transverse colon, thus resulting in the other pancreatic lymph node lacking

detectable accumulation of fluorescence under our IVIS setting. Intravenous administration allows particles to bypass intestine absorption and entering directly into circulation system, thus leading to observation of NLC accumulation in iLNs and both pLNs. No particles were detected in iLNs after oral delivery since those lymph nodes do not drain from intestine.

Figure 14. Lymph drainage along the length of the mucosa [69].



While treating Tofa-NLC alone provided promising therapeutic effect on delaying the onset of T1D diabetes, treatments with CTLA4-Ig gave negative results opposite to what we previously observed in the context of transplantation. The reason for costimulation blockade having different and even opposite effect in T1D is complicated, as it correlates to the activity of

multiple cell types as well as different pathways. Creating a balance between different cells and pathways is the key in developing new treatment, yet this is very difficult to manage and achieve.

CTLA4-Ig blocks the CD28 pathway by binding to CD80 and CD86 on APCs, preventing the activation of effector T cells (Teff), however, it can also affect other T cell subsets. For example, it can also block the generation and proliferation of regulatory T cells (Treg), whose development and homeostasis highly depend on CD28 pathway as well [70, 71]. Moreover, the interaction between CD28 and CD80/CD86 regulates the balance between type 1 T helper cells (Th1), which produces inflammatory cytokines in response to inflammation, and type 2 T helper cells (Th2), which produces cytokines essential for humoral responses and modulation of Th1 cell differentiation [72], and it has been shown in several autoimmune disease settings that CD4+ T cells differentiating towards Th2 is highly dependent on CD28 signaling, and disrupting CD28 pathway can lead to earlier onset and accelerated progressing of autoimmune disease [22]. Thus, the blockade of CD28 pathway can initiating more protective or more inflammatory responses by regulating cytokine balance, leading to positive or negative effects in T1D. Moreover, the existence of memory T cells (Tmem) can make co-stimulatory blockade less effective, as their activation threshold are much lower, thus less dependent on costimulatory signals and more resistant to costimulation blockade [73, 74]. The balance between Teff and Treg within pancreas as well as other secondary lymph nodes is very important in determining the ultimate fate of beta cells in the context of T1D, therefore, future experiments will investigate the Teff to Treg ratio.

Moreover, CTLA4-Ig can affect the signaling of other co-stimulatory pathways as well by interacting with different counter-receptors. For example, the blockade could interrupt the CTLA4 co-inhibitory pathway, which binds to the same ligands CD80/CD86 on APCs and plays

negative regulatory role by inhibiting T cell activation, reducing the expression of CD80/CD86, and increasing the expression of inhibitory molecule indoleamine 2,3-dioxygenase (IDO) [75, 76]. CTLA4 pathway is essential for immune homeostasis, and its expression on Treg is critical for their function [77, 78]. CD80 is also known to interact with programmed cell death 1 ligand 1 (PDL1), which provides inhibitory signal towards immune responses [79, 80]. Thus, blockade with CTLA4-Ig could affect not only CD28, but also CTLA4 and PD-L1 pathways, disturbing their inhibitory regulation on autoimmune responses, and leads to opposite outcomes eventually. Maybe future studies utilizing costimulation blockade as well as enhanced costimulation blockade strategy should choose blockade that binds to CD28 more specifically, such as anti-CD28 dAb and scFVs [81], in order to minimize the chance of interacting with other receptors and hopefully yield better outcomes.

The application of costimulation blockade was thought to be able to control graft rejection in transplant model by inducing T cell anergy, taking advantage of an acute phase during which foreign antigens are massively exposed to recipient upon surgery, leading to TCR engagement alone. However, autoimmune disease like T1D is a chronic disease and the development of autoreactivity spans much longer time-frame and remains quite elusive. There would be no moment of a massive presentation of autoantigens, yet before symptoms can be observed, autoantigens already exist. Thus, the negative effect CTLA4-Ig provided in our NOD model might also be due to lack of autoantigen-specific targeting, which raises the necessity of involving T1D autoantigen(s) (i.e. insulin) along with the enhanced costimulation blockade treatment to promote effective tolerance. Application of autoantigen, insulin-specific B peptide, is currently studied by our group in a separate project.

With all the characterization and elucidation of NLC particles as well as the NLC mediated delivery of enhanced costimulation blockade in T1D, future experiments should focus on studying the mechanism behind therapeutic effects from Tofa-NLC, exploring better treatment regimen according to any further characterization on NLC profile, and testing alternative costimulation/CD28 blockade options to better apply the enhanced costimulation blockade strategy in the context of type 1 diabetes.

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EDUCATION

- **The Johns Hopkins University** May, 2019
M.S. in Biomedical Engineering (expected)
The Whiting School of Engineering

- **The Pennsylvania State University, University Park** December, 2016
B.S. in Biology (Magna Cum Laude)
Eberly College of Science

ACEDEMIC EXPERIENCE

- **Research**

Department of Plastic and Reconstructive Surgery, Johns Hopkins School of Medicine
Vascularized Composite Allotransplantation (VCA) Lab January, 2018 --- Present
P.I.: Dr. Giorgio Raimondi
Project: *Lipid Nanoparticle-Mediated Delivery of Enhanced Costimulation Blockade (ECoB) to Promote Antigen Specific Immunotherapy of Type 1 Diabetes*

Department of Animal Science, The Pennsylvania State University, University Park
Advisor: Dr. Paul A. Bartell Aug. 2015—Dec.2016
Project: *The Regulation of Biological Clocks in Birds at the Systems Level*

- **Teaching**

TA for General Biochemistry (BMB401) at Penn State Fall 2016 Semester
TA for Introductory Physics II (PHYS 251) at Penn State Fall 2016 Semester

- **Conferences/ Presentations**

American Diabetes Association's 79th scientific sessions June, 2019
› Podium and Poster: *Lipid Nanoparticle-Mediated Delivery of Enhanced Costimulation Blockade to Prevent T1D*

IMMUNOLOGY 2019 (AAI annual meeting) May, 2019
› Podium and Poster: *Lipid Nanoparticle-Mediated Delivery of Enhanced Costimulation Blockade to Prevent T1D*

The 13th Annual Johns Hopkins Bayview Research Symposium December, 2018
› Poster: *Lipid Nanoparticle-Mediated Delivery of Enhanced Costimulation Blockade to Promote Antigen Specific Immunotherapy of Type 1 Diabetes.*

The Johns Hopkins Institute for Nanobiotechnology May, 2018
Annual Nano-Bio Symposium
› Poster: *Lipid Nanoparticle Platform for Therapeutic Delivery Applications*

The 10th annual Johns Hopkins Plastic and Reconstructive May, 2018
Surgery Research Symposium
› Podium: *Lipid Nanoparticle-Mediated Delivery of Enhanced Costimulation Blockade to Promote Antigen Specific Immunotherapy of Type 1 Diabetes.*